Investigation of the anti-breast cancer efficacy and mechanisms of disulfiram

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Abstract

Cancer is a major cause of morbidity and mortality affecting populations in all countries and all regions. Breast cancer (BC) is the second leading cause of cancer death among women in the UK. Although the overall survival of BC has been significantly improved due to systemic therapy in early BC, the treatment of advanced/metastatic BC remains a major challenge. The main limitation of therapeutic failure is the *de novo* and acquired resistance of BC cells to conventional anticancer drugs.

Cancer stem cells (CSCs) have been thought to be responsible for the chemoresistance. My study demonstrated that mammospheres manifested CSC characteristics and are highly resistant to several first line anti-BC drugs. This may be due to the hypoxia in the centre of the spheres. Transfection of BC cells with NFκB p65 induces CSC characters and chemoresistance. Therefore hypoxia-induced activation of NFκB could lead to escape of CSCs from apoptosis and regenerate the tumour after conventional chemotherapy. In clinic, the relapsed cancer is commonly pan-resistant to various drugs. Development of CSCs-targeting drug will be significantly important in clinic for cancer patients.

Disulfiram (DS) is a commercially available anti-alcoholism drug with strong cytotoxicity in a wide range of cancer types and has a reversing

effect on chemoresistance. In this study, the anticancer efficacy of DS on cancer cell lines and CSCs was investigated. DS was highly cytotoxic to BC cell lines *in vitro* in a copper (Cu)-dependent manner. CI-isobologram analysis demonstrated a synergistic effect between DS/Cu and paclitaxel (PAC) in BC cell lines. DS/Cu induces reactive oxygen species (ROS), activates JNK and p38 pathways and simultaneously inhibits NFκB activity in BC cell lines. DS/Cu may trigger intrinsic apoptotic pathway via modulation of the Bcl2 family. The *in vitro* clonogenicity and sphere-forming ability of BC cell lines were inhibited by DS/Cu. The common stem cell markers such as aldehyde dehydrogenase (ALDH) and $CD24^{-}$ /$CD44^{+}$ as well as Nanog, Sox2, and Oct4 were also suppressed. In PAC resistant cell line, DS abolished CSC characters and completely reversed PAC resistance. Lipo-DS blocked NFκB activation and specifically targeted CSCs *in vitro*. Lipo-DS also targeted CSC population *in vivo* and showed very strong anticancer efficacy.

This study elucidated the role of NFκB in bridging hypoxia with CSC-related chemoresistance. It also investigated the fundamental anticancer mechanisms of DS. The results derived from this study indicate that further study may be able to translate DS into cancer therapeutics in the future.

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Author's Declaration

I declare that the work in this dissertation is entirely my own, with the exception of the results presented in figures 5.5, which were produced in collaboration with Dr. Zhipeng Wang in the Fouth Military Medical University, China.

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Abbreviations

5-FU: 5-Fluorouracil

A/MBC: advanced/metastatic breast cancer

ABC: ATP-binding cassette

AJCC: American Joint Committee on Cancer

ALDH: aldehyde dehydrogenase

AML: acute myeloid leukemia

BAFF: B cell activating factor

BAX: BCL2-associated protein

BC: breast cancer

BCL-2: B-cell leukemia/lymphoma

BCRP: breast cancer resistance protein

BCSCs: breast cancer stem cells

BSA bovine serum albumin

CDDP: cisplatin

CDK: cyclin dependant kinase

CRUK: cancer research UK

CSC: cancer stem cell

Cu: copper

dCK: deoxycytidine kinase

DDC: diethyldithiocarbamic acid

DEPC: diethylpyrocarbonate

dFdC: 2 ′, 2 ′-difluorodeoxycytidine,

DMEM: Dulbecco's modified Eagle's medium

DNA: deoxyribonucleic acid

dNTP: deoxynucleoside triphosphate

Dox: doxorubicin

DS: Disulfiram

DTT: dithiothreitol

ECL: enhanced chemiluminescence

EMSA: electrophoretic mobility shift assay

EMT: epithelial to mesenchymal transition

ER: estrogen receptor

ERK: extracellular-signal related kinase

FCS: fetal calf serum

FLIP: FLICE-inhibitory protein

GAPDH: glyceraldehydes-3-phosphate dehydrogenase

GSH: oxidation of glutathione

GST: glutathione-S-transferase

H_2O_2: hydrogen peroxide

hCNT: the equilibrative (hENT) and the concentrative

HER2: human epidermal growth factor-2

HIF: Hypoxia inducible factor

HLH: helix-loop-helix domain

hNT: human nucleoside transporters

HRE: hypoxia-response elements

IKK: IκB kinase

IκB: Inhibitors of NFκB

JNK: c-Jun N-terminal kinases

LRP: lung resistance-related protein

MAPK: mitogen-activated protein kinase

MDR: Multiple drug resistance

MEK: MAPK/ERK kinase

MMPs: matrix metalloproteinases

MRP-1: multidrug resistance associated protein.

MTT: 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

NAC: N-acetyl cysteine

Nanog: homeodomain-containing transcription factor

NDDS: Nanotechnology-based drug delivery system

NFκB: Nuclear factor kappa B

NPC: nasopharyngeal carcinoma

NPs: Nanoparticles

Oct4: octamer-binding transcription factor 4

PAC: Paclitaxel

PARP: poly (ADP-ribose) polymerase

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

Pgp: P-glycoprotein

PHDs: prolyl-hydroxylases

PR: progesterone receptor

PVDF: Polyvinylidene difluoride

RHD: rel homology domain

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RR: ribonucleotide reductase

RRM1: Ribonucleotide reductase M1 polypeptide

RT-PCR: Reverse transcriptase polymerase chain reaction

SDS: sodium dodecyl sulphate

SDS PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

siRNA: small interfering RNA

SOD: superoxide dismutase

Sox2: SRY (sex determining region Y)-box 2

TAE: Tris-acetate-EDTA

TBST: Tris-tween buffered saline

TGF-β1: transforming growth factor β1

TNF: receptor-associated factors

TNM: Tumour, Node, and Metastisis

VEGF: vascular endothelial growth factor

VHL: von Hippel-Lindau

1. Introduction

1. 1 Cancer overview

Cancer is a serious worldwide public health problem characterized by uncontrolled growth of cells which cause serious problems by migrating from the original site and spreading to distant sites through the lymphatic system or bloodstream. It is a major cause of morbidity and mortality with approximately 14 million new cases and 8 million cancer-related deaths in 2012, affecting populations in all countries and all regions. It was responsible for 28% of all deaths in the UK in 2010, with around 77,400 more deaths due to cancer than coronary heart disease, and around 109,000 more deaths due to cancer than strokes (Sasieni *et al*., 2011; Boyle *et al*., 2003). The most commonly diagnosed cancers are breast, lung, colorectal and prostate cancers which account for over half of the cancer burden, and almost half of all cancer deaths are due to lung, bowel, breast and prostate cancers in the UK.

1. 2 Breast cancer

1.2.1 Incidence and mortality rates in the United Kingdom

Breast cancer (BC) is the most common female cancer in the UK. In 2010, 49,961 people were diagnosed with BC in the UK, an increase of 1.8% compared to 2009. These incidence rates have increased by 90% between 1971 and 2010(Statistical Information Team, CRUK, 2010).

BC is the second leading cause of cancer death after lung cancer since 1998 among women in the UK. In 2010, there were 11,633 deaths from BC; 11,566 (99%) of these were in women and 77 (1%) were in men (Statistical Information Team, CRUK, 2010). BC accounts for around 15% of female cancerous deaths in the UK (Office for National Statistics, 2011). A large proportion of BC cases in developed countries are related to reproductive and hormonal factors, obesity, alcohol and physical activity (Cancer Research UK, 2012). It is estimated that about 27% of cases of female BC in the UK are linked to largely modifiable lifestyle and environmental factors (Parkin *et al*., 2011). In the past 30 years, despite the major advances that have been made in understanding the biological and clinical nature of BC, the survival estimates of BC have risen steadily. However, it is still the most prevalent type of cancer among women in the developed world and its incidence has shown a continuous rise in recent decades.

Nowadays, BC has become a significant public health problem due to high mortality worldwide.

1.2.2 Classification of breast cancer

1.2.2.1 Histopathological and biological classification

Histopathological Classification: BC is a complex heterogeneous disease with each group having distinctive molecular variations, which can be characterized by different morphological, biological and behavioural features. These cases demonstrate a variety of unique characteristics, histological patterns and clinical outcomes (Weigelt *et al*., 2009). BC is a malignant tumour that originates from breast tissue, usually from either the inner lining of milk ducts (ductal carcinomas) or gland lobules (lobular carcinomas). Ductal carcinomas and lobular carcinomas account for the majority of these cases. However, the cancer can originate in other areas of the breast, and include comedo, tubular, and medullary carcinomas (Li *et al*., 2005). BC can be histopathologically subdivided into 4 categories, including invasive ductal BC, invasive lobular BC, inflammatory BC and less common types of BC (Cancer Research UK, 2012).

Biological Classification: New approaches have been considered to discover the molecular basis for the heterogeneity of BC. Molecularly defined classes of BC have been identified with distinctive biological and

clinical features using a special analysis of gene expression profiling. BC can be subdivided into three major biological subtypes using gene expression profiling by pathological features, including estrogen receptor positive (ER) and progesterone receptor positive (PR) and human epidermal growth factor-2 status (HER2) (Moulder *et al*., 2008). Further sub-classification of tumour types into luminal A (ER^+ and/or PR^+, $HER2^-$, low Ki67), luminal B (ER^+ and/or PR^+, $HER2^+$), human epidermal growth factor receptor-2 (HER2)(ER^-, $PR2^-$, $HER2^+$), and basal-like (ER^-, PR^-, and $HER2^-$) BCs (Perou *et al*., 2000; Sorlie *et al*., 2001)(Table 1.1).

Table 1.1 Molecular subtypes of breast cancer

Molecular Subtype	Representative Gene	Representative Immunophenotype	Histologic Features	Clinical Features
Luminal A	ESR1 (estrogen receptor1) Estrogen receptor-associated transcription factors	ER+ PR+ HER2- low Ki67	low grade	sensitive to endocrine therapy variable response to chemotherapy overall good prognosis
Luminal B	ESR1 (estrogen receptor1) Estrogen receptor-associated transcription factors	ER+ PR+ HER2+	higher grade than luminal A	sensitive to endocrine therapy variable response to chemotherapy prognosis poorer than that of luminal A
Basal-like	KRT5(keratin 5) KRT17 (keratin 17) LAMC2 (laminin, g2)	ER- PR- HER2-	high grade	insensitive to endocrine therapy variable response to chemotherapy overall poor prognosis
HER2	ERBB2 (HER2) ERBB2 amplicon	ER- PR- HER2+	high grade	respond to biologic therapy (trastuzumab) variable response to chemotherapy overall poor prognosis

(Perou *et al*., 2000; Sorlie *et al*., 2001)

1.2.2.2 Stages of breast cancer

The treatment decisions of BC patients depend on variables such as the site of the primary tumour, the size and number of tumours, cell type and tumour characteristics and grade. Classical number stages of BC system are commonly used in the UK. Details of this system are in Table 1.2. There is an American Joint Committee on Cancer (AJCC) staging system termed TNM (tumours/nodes/metastases) system. "T" denotes the degree of invasion of the intestinal wall, "N" denotes the degree of lymphatic node involvement, and "M" denotes the degree of metastasis. I will not discuss it in detail here.

Table 1.2 Number stages of breast cancer

Stage	Stage criteria
Stage 1A	The tumour is 2cm or smaller and has not spread outside the breast
Stage 1B	The tumour is 2cm or smaller and a few cancer cells in nearby lymph nodes
Stage 2A	1. No tumour or a tumour 2cm or smaller in the breast and the cancer cells in lymph nodes in either the armpit or near the breastbone. 2. The tumour is 2-5cm and there is no cancer in the lymph nodes
Stage 2B	1. The tumour is 2-5cm and a few cancer cells in nearby lymph nodes. 2. The tumour is 2-5cm and the cancer cells in 1-3 lymph nodes in either the armpit or near the breastbone. 3. The tumour is larger than 5cm but not in the lymph nodes.
Stage 3A	1. The tumour is of any size and the cancer cells in 4-9 lymph nodes. 2. The tumour is larger than 5cm and a few cancer cells in nearby lymph nodes. 3. The tumour is larger than 5cm and the cancer cells in 1-3 lymph nodes in either the armpit or near the breastbone.
Stage 3B	1. The cancer has spread to chestwall. 2. The tumour has spread to the skin of the breast.
Stage 3C	1. The tumour can be any size and the cancer cells in 10 or more lymph nodes in the armpit. 2. The tumour can be any size and the cancer in lymph nodes above or below the collar bone. 3. The tumour can be any size and the cancer in lymph nodes in the armpit and near the breastbone.
Stage 4	The tumour can be any size and the cancer has spread (metastasised) to other parts of the body such as the bones, lungs, liver or brain

(Cancer Research UK, 2012)

1.2.3 Therapeutic of breast cancer

In clinical, four main treatment options such as surgery, radiotherapy, chemotherapy and molecular-based therapy will be available for the BC depends on different requiring of patients.

1.2.3.1 Surgery

Surgery is commonly the first treatment for BC which has not spread to the axillary lymph nodes. Surgical excision has been reduced to preserve most breast tissue in women suffering from stage I and II BC during the last 30 years (Lakhani *et al*., 2006). With the development of lymph node mapping and biopsy techniques, many new cases will be diagnosed in the early stages of BC (Lakhani *et al*., 2006), therefore, patients may be offered a test to detect one or two critical sentinel lymph nodes at the beginning, but if cancer cells have already been detected in most lymph nodes by biopsy, an axillary dissection will be applied to surgically remove all the lymph nodes.

1.2.3.2 Radiotherapy

Radiotherapy is an important treatment to destroy cancer cells using high energy x-rays. Regular treatment will be given to achieve the greatest effect on the cancer cells while limiting the damage to normal cells. Radiotherapy is used to treat BC in different ways by eliminating any remaining cancer

cells in the breast where the cancer has been removed after surgery, including breast conserving surgery (a wide local excision or lumpectomy) or mastectomy. Moreover, radiotherapy can efficiently reduce the risk of cancer recurrences through treating the lymph nodes in the armpit which may still contain cancer cells and above the collarbone depending on the surgery (Clarke *et al*., 2005). Radiotherapy is most commonly used before, after or during courses of chemotherapy.

1.2.3.3 Endocrine therapy and biological treatments

BC cells are assessed for biological markers (ER, PR, and HER2). Around 70% of BC cases overexpress ER and 15-20% of invasive BCs overexpress HER2. ER and HER2, when bonded by their natural ligand, would promote cell proliferation and differentiation therefore promoting cancer development (Lewis *et al*, 2002; Ward *et al*, 2009; Hind *et al*, 2007). Endocrine therapy is the treatment of BCs with additional hormone drugs, after surgery, in order to prevent cancer relapse. This treatment can block the signalling pathway between oestrogen and ERα by either interfering with ER-ligand interaction using tamoxifen which is one of the selective ER modulators (SERMs) or decreasing production of the ligand through ovarian ablation or suppression (OA/OS) or aromatase inhibition (Bao *et al*., 2007). Studies show that the use of endocrine therapy would effectively reduce BC recurrence rates by 40% and death rates by 30% (Statistical

Information Team, CRUK, 2009). Endocrine therapy represents the first targeted therapy to treat BC patients with the ER^+ type of BC. Moreover, cancer cells that express ER and HER2 are more aggressive and are associated with increased risk of recurrence and death. Anti-HER2 antibody (trastuzumab) is used to treat the BC cells with the $HER2^+$. The anti-HER2 antibody would bind to HER2, blocking the ligand binding site and inhibiting signal transduction. Studies showed that treating a $HER2^+$ BC patient with trastuzumab would reduce the risk of relapse by 50% and death by 30% (Lewis *et al*, 2002; Ward *et al*, 2009).

1.2.3.4 Chemotherapy

Chemotherapy is a treatment using a drug or combination of drugs which is cytotoxic to rapidly dividing cells, such as cancer cells but also normal cells. The adjuvant chemotherapy can be used as an additional therapy before or after surgery and/or radiotherapy for primary invasive BC. Chemotherapy can be widely offered based on different factors including age and the size or grade of BC in patients（Howell et al., 2005）. When the BC cells have spread from the breast to other places, chemotherapy can be used for controlling the growth of metastatic BC. It may also reduce some symptoms caused by the metastatic BC. Cancer cells always grow in a disordered and uncontrolled way. The drugs work by disrupting the growth of cancer cells but the mechanisms of different chemotherapy drugs

are not the same. The chemotherapy drug targets cancer cells via the circulation of the blood in the body. Normal cell growth is a tightly controlled process coupled with cell death (apoptosis) to prevent excessive growth. The growth of cancer cells is out of control coupled with inhibition of apoptosis. Chemotherapy inhibits cancer cell proliferation and induces cancer cells into apoptosis.

In stage I BC cases, chemotherapy is used only for patients who are assessed to have a high risk of recurrence because of the side effects associated with chemotherapy drugs. The standard chemotherapy drugs used are anthracycline-based regimens such as epirubicin or doxorubicin (Dox) in clinic (Lister-Sharp *et al*, 2000; Takeda *et al*, 2008; McArthur *et al*, 2007). In stage II/III cases, where cancer cells are found in axillary lymph nodes, the patients would usually be treated with standard chemotherapy treatment followed by paclitaxel (PAC) or docetaxel. In stage IV cases, patients are treated with a larger selection of chemotherapy drugs, including the standard epirubicin or dFdC, PAC or docetaxel, platinum agents, cyclophosphamide, vinorelbine, nucleotide analogue and ixabepilone (Takeda *et al*, 2008; Jones *et al*, 2004; Ross *et al*, 2004; McArthur *et al*, 2007).

More than 50 different anti-cancer drugs are available in clinic. These chemotherapeutic agents can be categorized as antimetabolites,

anthracyclines, plant alkaloids (Taxanes), alkylating agents, antitumour antibiotics, monoclonal antibodies and platinums (Raina *et al*., 2007). Many of them have been used in advanced BC treatment. Table 1.3 lists the anticancer drugs most commonly used in BC chemotherapy. In this section, I will focus on three first line anti-BC drugs which I will examine in my study (dFdC, Dox and PAC).

Table 1.3 Drugs commonly used in breast cancer chemotherapy

Drug	Response rate (%)
Doxorubicin	10–50%
Gemcitabine	12–37%
Paclitaxel	21 days: 16–62% 7 days: 22–53%
Epirubicin	13–48%
Liposomal doxorubicin	10–50%
Cisplatin/carboplatin	9–51%
Docetaxel	21 days: 18–68% 7 days: 33–50%
ABI-007 (Abraxane)	33–48%
Capecitabine	20–35%
Vinorelbine	25–50%

(Takeda *et al*, 2008; Jones *et al*, 2004; Ross *et al*, 2004; McArthur *et al*, 2007)

1.2.3.4.1 Gemcitabine

Gemcitabine (2', 2'-difluorodeoxycytidine, dFdC) (Fig. 1.1) is a deoxycytidine analogue with proven anticancer activity both *in vitro* and *in vivo*, which was first synthesized in the 1980s by Eli Lilly Inc., Indianapolis, as an antiviral agent (Bergman *et al*., 2005). It is able to elicit chemotoxic activity upon a broad range of cancers. In clinical practice, it is an agent used to treat patients with advanced metastatic pancreatic cancer and in combination therapy for non-small cell lung cancer, bladder, ovarian and BC (Mini *et al*., 2006).

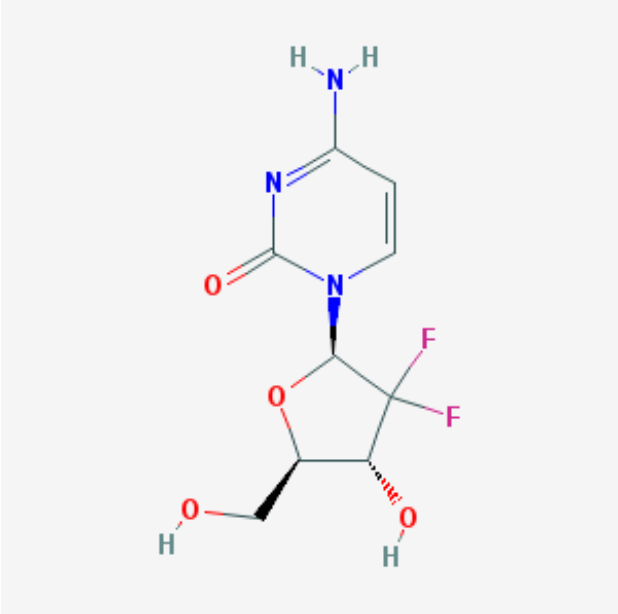


Fig. 1.1 Structure of Gemcitabine (Image from PubChem)

dFdC is a prodrug requiring intracellular phosphorylation to become active. Due to the hydrophilic nature of the molecule, specialised human nucleoside transporters (hNT) are required to facilitate the movement of the

molecule. These transporters are divided into two groups: the equilibrative (hENT) and the concentrative (hCNT) types (Shah *et al*., 2007). The hENT1 appeares to be the major transporter among the main dFdC transporters including hENT1, hENT2, hCNT1 and hCNT3. Once dFdC enters into the cell, it undergoes phosphorylation by dCK to dFdC monophosphate (dFdCMP), this is followed by further phosphorylation by nucleoside monophosphate kinase and nucleoside diphosphate kinase producing active dFdC diphosphate (dFdCDP) and triphosphate (dFdCTP) respectively (Ueno *et al*., 2007; Galmarini *et al*., 2004). The cytotoxic action of dFdC is mediated by these phosphorylated derivatives. dFdC diphosphate is able to indirectly inhibit DNA synthesis via inactivation of ribonucleotide reductase (RR), which is an enzyme essential for the conversion of RNA nucleotides to DNA nucleotides. This process results in the "self-potentiation" of dFdC as the concentration of naturally occurring deoxynucleotide triphosphates is reduced (Heinemann *et al*., 1992).

It is believed that incorporation of dFdC triphosphate into the DNA strand is the major mechanism by which dFdC induces apoptosis, specifically acting upon S and G1/S phases of the cell cycle (Heinemann *et al*., 1992). Once dFdC triphosphate is incorporated into the DNA strand, DNA polymerases are unable to proceed. This process ("masked termination") protects the drug, as proofreading enzymes are unable to remove dFdC

from this position. Overall, the unique actions that dFdC metabolites exert on cellular regulatory processes along with dFdC triphosphate incorporation in the DNA serve to inhibit cell growth and induce cell death (Heinemann *et al*., 1992).

1.2.3.4.2 Doxorubicin

Dox is a member of the anthracycline drug family which is isolated from different strains of Streptomyces in the 1970s (Woodruff *et al*., 1960) (Fig. 1.2) and is widely used in the chemotherapy of solid tumours, lymphomas and leukaemia (Minotti *et al*., 2004). It is considered as the most effective therapeutic agent for BC chemotherapy (Smith *et al*., 2006).

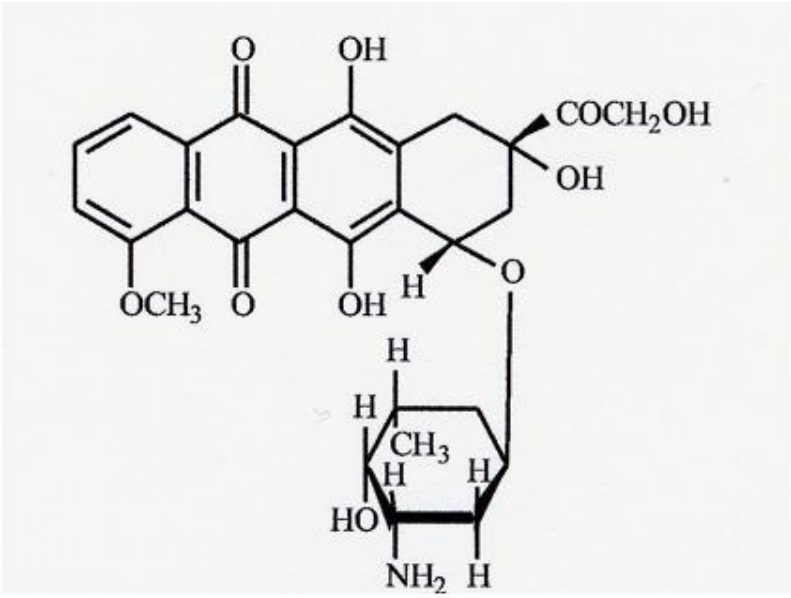


Fig. 1.2 Structure of Doxorubicin (Smith *et al*., 2000)

The anti-cancer mechanisms of Dox are multifactorial. There are two proposed mechanisms of Dox acting in the cancer cell. It is believed that

Dox intercalates into DNA and disrupts topoisomerase-II-mediated DNA repair and the other mechanism is that Dox can generate free radicals resulting in damaging to cellular membranes, DNA and proteins. Once Dox enters the cancer cells, firstly, it binds to DNA by inserting between the stacked paired bases of the super-coiled double helix. This process leads to uncoiling of the helix resulting in impairment of topoisomerase II activity. Topoisomerase II is an essential enzyme in controlling the supercoiling of DNA playing an important role in maintaining DNA structure by creating and repairing nicks in two adjacent parent DNA strands (D'Arpa et al., 1989). When it has been inhibited, DNA replication and transcription will not work properly and results in permanent DNA cleavage (D'Arpa *et al*., 1989). Dox may also alter membrane fluidity and affect ion transport and hence biochemical equilibria by binding to cell membrane. Dox induces DNA damage by generating semi quinone species that leads to the production of free radicals and hydroxyl radicals (Adama *et al*., 2003). Proteasomes have been reported to modulate the activity of Dox by degrading proteins through non-lysosomal mechanisms. The 26S proteasome has been shown to control an increasing number of essential biochemical mechanisms of the cellular lifecycle including processing and degradation of regulatory proteins (Adama *et al*., 2003; Cusack *et al*., 2003). Dox is transported to the nucleus of the cells by the proteasome through a series of steps. After getting into the cancer cells, Dox

automatically binds to the 20S proteasomal subunit in the cytoplasm. This complex was driven into the nucleus through nuclear pores by an ATP-dependent process. Dox shows its higher affinity to DNA resulting in dissociation from the proteasome and binding to DNA (Kiyomiya *et al*., 2001). The final apoptosis signal will appear after accumulation of the degraded proteins.

1.2.3.4.3 Paclitaxel

PAC is derived originally from *Taxus Brevifolia* (Bark of Pacific yew/Western yew conifers) and it was discovered to be as an anti-leukemic agent in 1971 (Wani *et al*., 1971). In the past few years, as the first line anti-cancer drug in the clinical treatment, PAC has been successfully used to treat a range of human cancers including ovarian, metastatic breast and non-small cell lung carcinomas (Rowinsky *et al*., 1992). Due to a shortage of enough Taxus bark, this drug cannot be widely used for the patients until scientists have successfully developed semisynthetic derivatives based on the molecule structure of paclitaxel (Fig. 1.3).

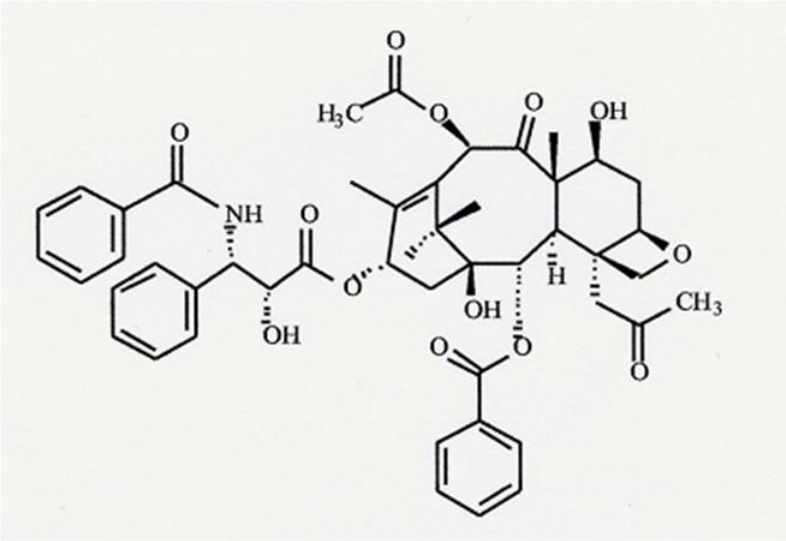


Fig. 1.3 Structure of Paclitaxel (Smith *et al*., 2000)

It is well known that PAC can suppress and inhibit cell growth, differentiation and proliferation in many cancer cell lines and it is the most preferred anti-cancer drugs by clinical doctors. PAC works completely differently from dFdC and Dox. The main mechanism of action of PAC is stabilising the microtubules and inhibiting the anaphase of the cell cycle. The stabilisation of the microtubules in anaphase would prevent separation of the chromosome which is essential for division of the parent cell into two daughter cells. The dynamic polymerisation and de-polymerisation of microtubules is essential for the normal function of cellular processes including chromosome separation and cytokinesis. A microtubule is a cylindrical assembly of tubulin dimers which are composed of α and β-tubulin subunits (Chretien *et al*. 1992). The β-tubulin subunit is known to have the binding site for PAC. When PAC is binding to the β-subunit of

tubulin, the formation of structurally abnormal microtubules is relatively resistant to de-polymerisation. The subunits of microtubules are abnormally stabilized by PAC (Bharadwaj *et al*., 2004; Brito *et al*., 2008; Ganguly *et al*., 2010).

1.3 Chemoresistance

Chemotherapy is one of the principal treatments for advanced and metastatic BC. Drug resistance is the main limitation to the success of chemotherapy. Inadequate drug exposure, insufficient dosage, poor tumour vascularisation and poor drug bioavailability and distribution are the main causes of drug resistance during chemotherapy. Intrinsic and acquired drug resistance are the two main categories of chemoresistance (Longley *et al*., 2005). Some cancer cells seem to be inherently resistant to anticancer drugs (Longley *et al*., 2005). There are a range of molecular mechanisms implicated in drug resistance, including multidrug resistance (MDR), DNA damage repair, CSCs, and tumour microenvironment.

Cancer cells displaying reduced sensitivity to drugs through continued exposure can also become cross-resistant to other unrelated classes of chemotherapeutic drugs, which is known as MDR. A few kinds of chemotherapy drugs such as PAC and Dox could have totally different structures and mechanisms of cytotoxicity, but they share a common

transport mechanism which is controlled by ATP-binding cassette (ABC) transporter family (Moscow *et al*., 1988). Drugs are actively pumped out of the cell as a consequence of expression of ABC transporter proteins. The ABC transporter proteins are a large superfamily of membrane proteins comprising 49 members divided into seven different families based on sequence similarities (Moscow *et al*., 1988; Gottesman *et al*., 1993). Overexpression of the *MDR1* gene in multidrug resistant cancer cells leads to a large production of MDR1 protein (P-glycoprotein (Pgp). The anti-cancer drugs were pumped out from the cytosol to the extracellular medium by using the energy of ATP hydrolysis. Pgp consistently plays the role of drug removal and expression on some functional barriers including kidney tubules, liver cells and blood tissue barriers, and it also has key functions in immunology and apoptosis by selective expression in the adrenal gland, haematopoietic stem cells, and T and B lymphocytes (Moscow *et al*., 1988; Gottesman *et al*., 1993). Some other members of the ABC transporter proteins superfamily, including multidrug resistance associated protein (MRP-1), lung resistance-related protein (LRP) and breast cancer resistance protein (BCRP) also show their special importance in drug resistance.

A few chemotherapeutic drugs can induce DNA damage either directly or indirectly. The cellular response to DNA damage is repair or cell death. Therefore, the ability of DNA damage repair in cancer cells has a major

influence on the effectiveness of drugs. DNA damage slows down the cell cycle allowing cells to repair the damage. In some cancers, functional alteration of oncogenes and/or suppressor genes can disrupt the regulation of cell cycle arrest. For example, p53 plays an important role in regulating numerous cell cycle checkpoints. Mutation of p53 can disrupt DNA-damage-induced cell cycle arrest, and is frequently associated with drug resistance (Tergaonkar *et al*., 2002; Webster *et al*., 1999).

Beside the cellular response, cancer microenvironment is considered to be another key factor in the initiation and maintenance of drug resistance. More recently, a new research paradigm suggesting the role of CSCs in the intractability of cancers has been discussed world-wide. CSCs have been found to express ABC family members such as BCRP and other ABC efflux pumps (Victor *et al*, 1997). This allows them to survive hypoxia and the effects of multiple chemotherapeutic drugs, and it has been also found that CSCs can initiate another tumour and differentiate so that the new tumour contains populations of CSCs and non-stem cells. There are still some other mechanisms to explain the drug resistance, including expression of important apoptosis-associated proteins such as BCL-2 family (Reed *et al*., 1995; Clynes *et al*., 1998), the tumour suppressor protein p53 (Mueller *et al*., 1996), and dysfunctional metabolism of ceramide. Another common mechanism of resistance in cancer cells is

Glutathione up-regulation. This endogenous antioxidant is believed to cause resistance by providing protection against free radical damage (Lutzky *et al*., 1989). It relies on the ability of resistant tumour cells to efficiently promote glutathione S-transferase (GST)-catalyzed oxidation of glutathione (GSH) conjugation of anti-tumour drugs (O' Brien *et al*., 1996).

Three anticancer drugs (dFdC, PAC and Dox) were used in my study. The resistant mechanisms of these drugs are listed in Table 1.4.

Table 1.4 Resistance mechanisms of chemotherapeutic agents

Cytotoxic agent	Cancer type	Target	Resistance mechanism
Gemcitabine	Breast cancer Colorectal cancer Pancreatic cancer Gastric cancer Head and Neck cancer Ovarian cancer Lymphoma Leukaemia	Thymidylate synthase DNA synthesis	Increased thymidylate synthase expression MLH1 hypermethylation Activation of survival pathways Increased expression of anti-apoptotic proteins
Paclitaxel	Breast cancer Lung cancer Ovarian cancer Head and Neck cancer Kaposi's sarcoma	Microtubule Tubulin	Tubulin mutations MDR1 overexpression Chromosomal instability
Doxorubicin	Breast cancer Kaposi's sarcoma Ewing's sarcoma Lung cancer Testicular cancer Lymphoma Leukaemia Glioblastoma	Topoisomerase II	MDR1 overexpression Mutation or decreased expression of topoisomerase II Decreased apoptosis due to mutation of p53

(Bharadwaj *et al*., 2004; Heinemann *et al*., 1992; Kiyomiya *et al*., 2001)

1.4 Nuclear Factor Kappa B

The first member of nuclear factor kappa B (NFκB) protein family was discovered by Ranjan Sen and David Baltimore in 1986 as a factor in the nucleus of B cells that bound selectively to the enhancer of the kappa light

chain of immunoglobulin (Sen *et al*., 1986). Over 25 years of intense research demonstrated that NFκB transcription factor is ubiquitously expressed in almost all cell types or tissues, and regulates the expression of a large variety of genes which have NFκB binding site in their promoters/enhancers. It is now elucidated that NFκB plays a critical role in mediating responses to a remarkable diversity of external stimuli and is involved in complex biological processes such as inflammatory and immune responses of the cell, cell growth and development, pathogens, injuries, and other stressful conditions. Therefore, it is a pivotal element in multiple physiological and pathological processes (Oeckinghaus *et al*., 2009). NFκB proteins have the ability to influence expression of numerous genes, so the activity of NFκB is tightly regulated at multiple levels.

1.4.1 The structure of NFκB family members

The NFκB family in mammalian cells consists of five structurally related and functionally conserved proteins: RelA (p65), RelB, c-Rel, NFκB1 (p50/p105), and NFκB2 (p52/p100) (Frances E *et al*., 1999) (Fig. 1.4). All components share a highly conserved 300 amino acid sequence called Rel homology domain (RHD) at their N-termini, which is primarily responsible for DNA binding specificity, the C-terminal domain of this region is responsible for dimerization and IκB binding. Based on the synthesis and transactivation properties of sequences from C-terminal to the RHD, NFκB

proteins are divided into two classes. The class I NFκB proteins include NFκB1 and NFκB2 which are synthesized as large precursors (p105 and p100, respectively) with an N-terminal RHD and a long C-terminal domain that contains multiple copies of inhibitory series of ankyrin repeats. The processing of p105 and p100 is mediated by the ubiquitin/proteasome pathway and removes the C-terminal domain, resulting in production of the mature DNA-binding proteins (p50 and p52) that lack a transactivating domain. The class II NFκB proteins consist of RelA (p65), RelB and c-Rel which are synthesized in their mature forms and contain C-terminal transcription activation domains but without the inhibitory ankyrin repeats (Karin *et al*., 2002). In unstimulated cells, NFκB dimers are located in the cytoplasm in an inactive form as a consequence of their association with members of another family of proteins called IκB (inhibitors of κB) (Karin *et al*., 2002). The IκB inhibitory proteins also form a family containing seven members in mammals including IκBα, IκBβ, IκBε, IκBγ, Bcl-3, and the carboxyl-terminal regions of NFκB1 (p105) and NFκB2 (p100) (Fig. 1.4). The IκB proteins are characterized by the presence of multiple ankyrin repeats at their C-terminal domains through which they associate with the RHD of NFκB proteins (Momoko *et al*., 2005). This interaction retains NFκB in the cytoplasm. Class I NFκB proteins generally keep inactive, when they form dimers with members of class II NFκB proteins, the homodimers or heterodimers transcription will start by binding to 9-10 base

pairs DNA sites which are called κB sites (kappaB sites, 5'-GGGRNNYYCC-3'; R, purine; Y, pyrimidine; N, any nucleotide)(Momoko *et al*., 2005).

The NFκB family members form various homo/heterodimers that recognize and bind to similar DNA sequences and regulate the expression of distinct sets of genes. In most mammal cells, the major Rel/NFκB complex is the heterodimer of the p65/RelA and p50 subunits. p50 and p52 play critical roles in modulating the specificity of NFκB function. In contrast to RelA, RelB and c-Rel, no transactivation domains are located in the C terminal halves of p50 and p52 NFκB subunits. In general, homodimers of p50 and p52 inhibit κB site transcription, but both p50 and p52 participate in targeting gene transactivation by forming heterodimers with RelA, RelB or c-Rel (Li *et al*., 2002). The diversity of Rel/NFκB proteins shows distinct abilities to form different dimers, binding to different κB sites and IκB inhibitor proteins (Barkett, *et al*., 1999). In different cell types, Rel/NFκB complexes can be induced with other transcription factors and regulatory proteins to regulate the expression of distinct gene sets.

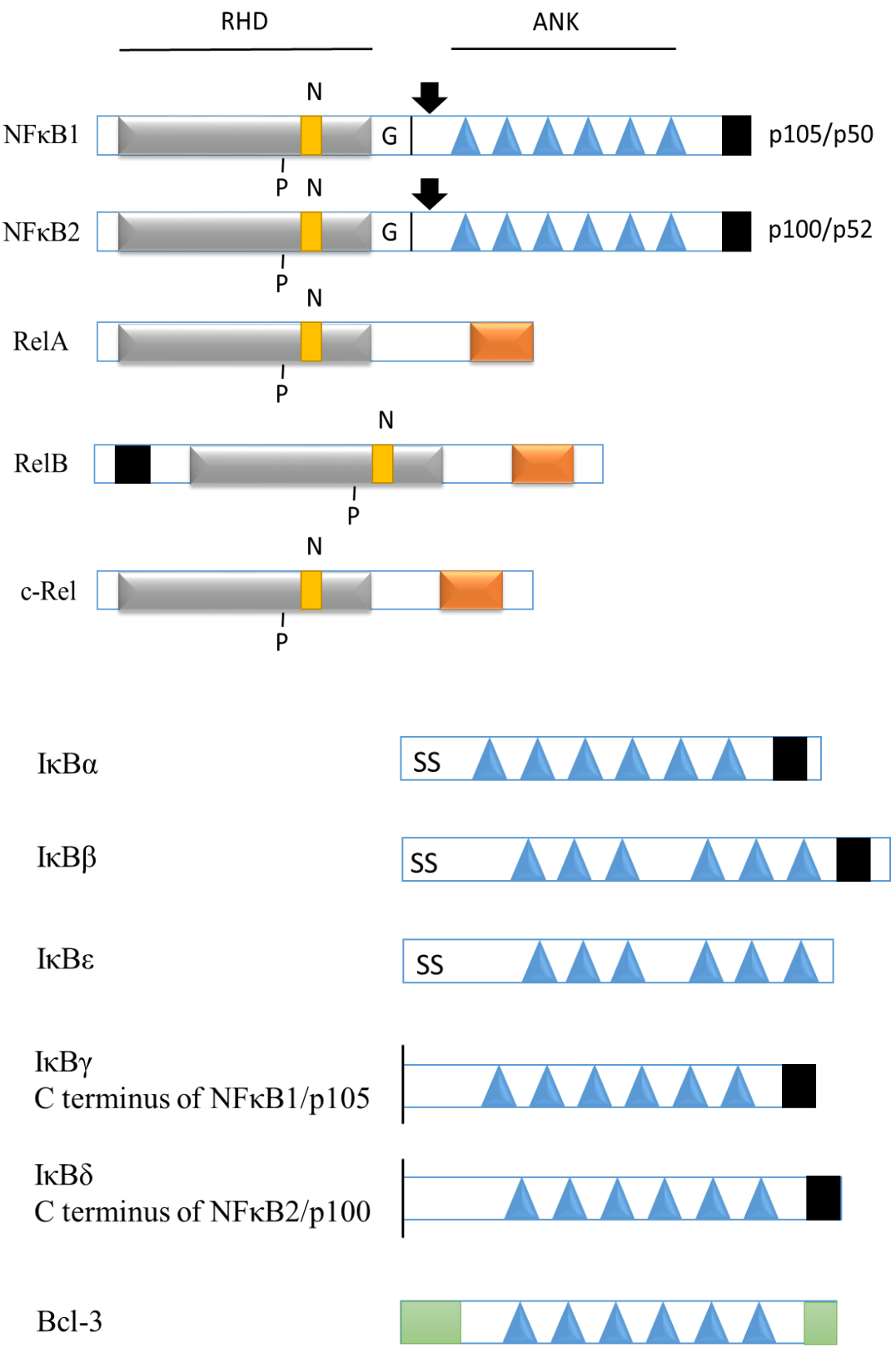


Fig. 1.4 Members of the Rel/NFκB and IκB families of proteins. The arrows indicate the endoproteolytic cleavage sites of p105 and p100 which give rise to p50 and p52, respectively. Black

boxes indicate the PEST domains (polypeptide sequences enriched in proline, glutamic acid, serine, and threonine), green boxes on Bcl-3 indicate transactivation domains, orange boxes on RelB indicate leucine zipper domains, and yellow boxes indicate nuclear localization sequence (NLS). Abbreviations: RHD, Rel homology domain; ANK, ankyrin repeat; SS, signal-induced phosphorylation sites. N, nuclear localization sequence.

1.4.2 The activation of NFκB pathways

In normal quiescent cells, NFκB remains in the cytoplasm in an inactive form by binding to IκB proteins (Ghosh *et al*., 2002). Degradation of IκB is a tightly regulated event that is initiated by specific phosphorylation of IκB proteins via activation of the IκB kinase (IKK). The IκB kinase enzyme complex is part of the upstream NFκB signal transduction cascade. As a 700-900 kDa complex, the IKKs complex consists of two catalytic kinase subunits, IKKα (IKK1) and IKKβ (IKK2), and a regulatory subunit IKKγ, also termed NEMO (NFκB essential modulator)(Ghosh *et al*., 2002). IKKα and IKKβ are 52% identical and form homodimers or heterodimers. IKK complex, residing at a key convergence site, leads to NFκB activation in multiple signalling pathways. IKKα and IKKβ are expressed everywhere and consist of an amino-terminal kinase domain, a leucine zipper, and a carboxy-terminal helix-loop-helix domain (HLH) (Ghosh *et al*., 2002).

There are two IKK-dependent signalling pathways leading to NFκB activation: classical and the alternative pathway (Fig. 1.5). In the classical NFκB signalling pathway, it is triggered when the cells have been

stimulated by TNFα, T-cell receptors (TCR), B-cell receptors (BCR), Toll-like receptor (TLR) or interleukin-1 receptor (IL-1R) superfamily members (Karin *et al*., 2000). IKKβ is both necessary and sufficient for phosphorylation of IκBα on Ser32 and Ser36, and IκBβ on Ser19 and Ser23 (Karin *et al*., 2000). The role of IKKα in the classical pathway is not confirmed, but recent studies suggest it may regulate gene expression in the nucleus by modifying the phosphorylation status of histones (Karin *et al*., 2000). The phosphorylation and activation of IKKβ can be induced by the stimulating factors. IκB proteins are phosphorylated by activated IKK complex at Ser32 and Ser36 of IκBα. Phosphorlation triggers polyubiquitination at sites equivalent to Lys21 and Lys22 of IκBα and degradation by 26S proteasome, releasing free NFκB (RelA/p50) dimmers to the nucleus (Karin *et al*., 2000). The alternative pathway depends only on the IKKα subunit, which functions by phosphorylating p100 and causing its inducible processing to p52. It is involved in the specific activation of RelB/p52 heterodimers. The p52/p100 protein binds to amino-terminal of RHD in RelB and keeps the dimer in the cytoplasm. The activated IKKα dimers induce the degradation of p100 and results in the nuclear entry of RelB/p52 heterodimers. The alternative pathway is activated in response to a subset of NFκB inducers including LTβ and lymphotoxin B and B cell activating factor (BAFF) and CD40L via NFκB inducing kinase (NIK) (Bonizzi *et al*., 2004).

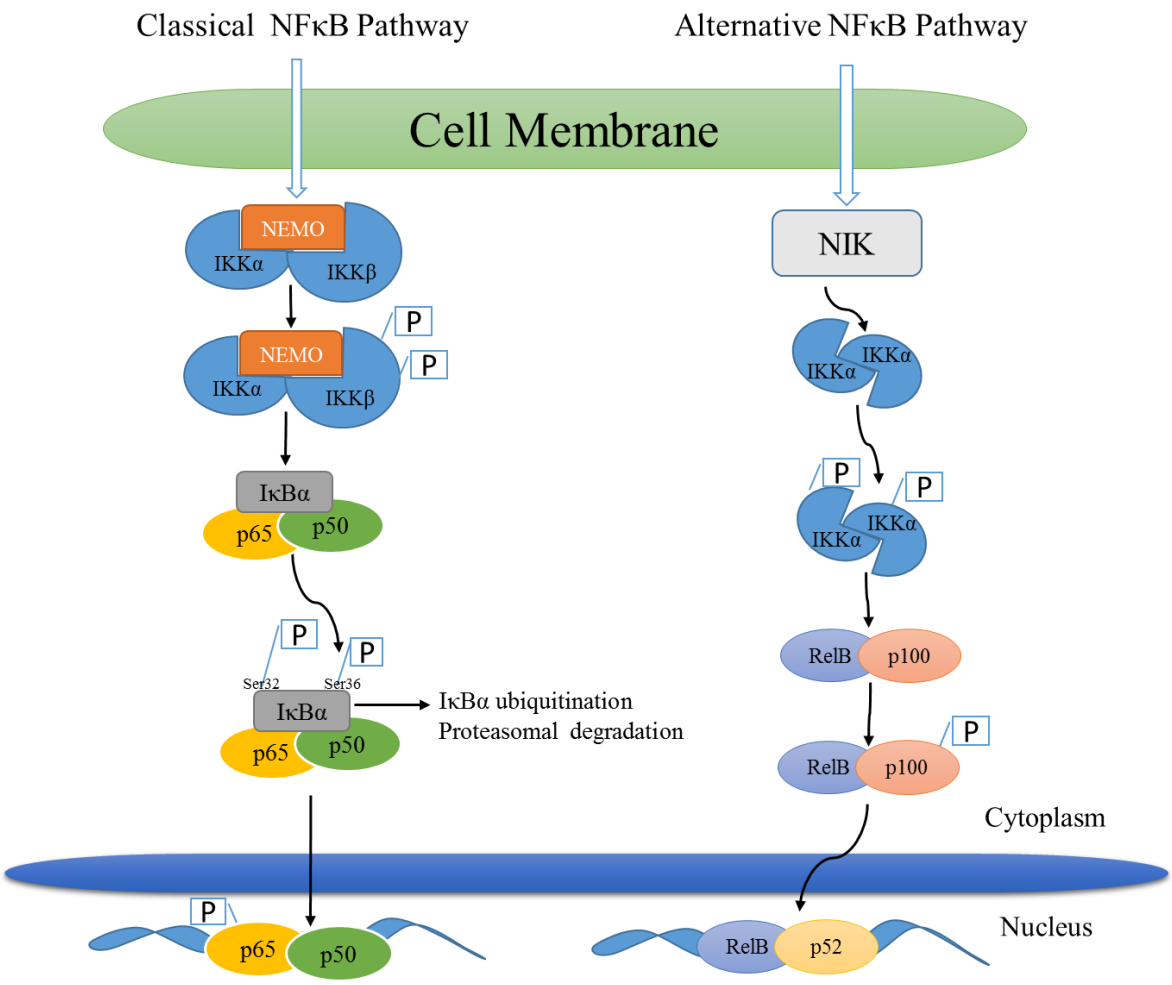


Fig. 1.5 Classical and alternative pathways of NFκB activation. The classical pathway mediated by IKKβ and leading to phosphorylation of IκB. Inputs for the classical pathway include TNFR1/2, TCR and BCR, TLR/IL-1R, and many others. The alternative pathway involves NIK activation of IKKα and leads to the phosphorylation and processing of p100, generating p52: RelB heterodimers. Input signals for the alternative pathway follow ligation of LTβR, BAFFR, and CD40R. These alternative pathway stimuli also activate the classical pathway.

1.4.3 NFκB and cancer

NFκB is a key factor in controlling both innate and adaptive immunity. The constitutive activation of NFκB pathways is often associated with inflammatory diseases. In 1858, Virchow suggested that chronic

inflammation might give rise to malignancy. Afterwards, accumulating data demonstrated that many types of cancers have been involved with chronic inflammation (Coussens *et al*., 2002). The animal study data demonstrated the important role of NFκB activation in tumour development. Deleted IKKβ in intestinal epithelial cells leads to a significant reduction in tumour incidence in a mouse model of colitis-associated cancer, whereas knocking out IKKβ in myeloid cells in these mice results in decreased tumour size by diminished expression of pro inflammatory cytokines that act as tumour growth factors (Coussens *et al*., 2002). Moreover NFκB plays a critical role in cancer progression by controlling epithelial to mesenchymal transition (EMT) and metastasis which is often associated with an up-regulation of matrix metalloproteinases (MMPs), freeing the extracellular matrix for evasion of cancer cells (Huber *et al*., 2004). NFκB can also up-regulate vascular endothelial growth factor (VEGF) and its receptors to control vascularization in tumour progression (Xie *et al*., 2010; Yoshida *et al*., 1999). NFκB is important for survival of cancer cells and mediates aggressive tumour phenotype. It is widely accepted that NFκB in particular has a double-edged role in cancer. On one hand, constitutive activation of NFκB can induce a variety of pro-tumorigenic functions in many types of cancer. On the other hand, as a part of the immune defending system, activation of NFκB could lead to the targeting and elimination of transformed cells and involve for acute inflammatory

processes with a high activity of cytotoxic immune cells against cancer cells (Disis *et al*., 2010).

Understanding the activity and function of NFκB in the context of tumourigenesis may provide opportunities for the development of new treatments to inhibit prolonged activation of this pathway and this is an important challenge for modern cancer biology.

NFκB and Breast Cancer: Over the past 20 years, there have been significant gains in the understanding of the molecular events associated with BC development and therapeutic resistance. NFκB has been recognized as an upstream regulator of many important cellular signalling pathways, including those related to BC promotion. Furthermore, NFκB is also an important modulator of the apoptotic response of cells to injury. Regulation of the apoptotic pathway by NFκB may affect both the pathogenesis of BC and its response to chemotherapy and radiation. NFκB protein usually up-regulates anti-apoptotic genes resulting in the provision of a cell survival mechanism to withstand drug treatment in BC.

BC cells have been shown to express constitutively active NFκB through abnormal signalling via the epidermal growth factor receptor, interleukin-1, or heregulin pathways (Bhat-Nakshatri *et al*., 2002). There is a cross-talk between the NFκB and ER pathways that have been found to get involved in metastasis and give a worse prognosis for patients with ER negative BCs

(Nozaki *et al*., 2000). The level of osteoclastogenic factors have been induced by constitutive activation of NFκB through the receptor activator of NFκB pathway, resulting in the promotion of extensive bone loss within BC bony metastases (Lee *et al*., 2003). The cellular microenvironment may affect the expression of NFκB. Some growth factors and hypoxic condition within the tumour microenvironment may contribute to constitutive NFκB activity in BC (Kim *et al*., 1999). Finally, a positive feedback loop will start when NFκB protein is expressed aberrantly, leading to the expression of more NFκB protein (Kim *et al*., 1999).

There is much evidences showing that NFκB DNA-binding and transcriptional activity is at a very high level in both mammary carcinoma cell lines and primary human BC tissues (Biswas *et al*., 2001). Furthermore, High IKK kinase activity has been detected in transformed BC cell lines and in primary human BC specimens, thereby NFκB activity will be decreased by inhibition of IKK activity in tumour cell lines (Deng *et al*., 2002). The transforming growth factor β1 (TGF-β1) inhibits mammary tumour cell growth by the inhibition of aberrantly activated NFκB activity (Sovak, *et al*., 1999). Although much evidences suggests that NFκB is implicated in BC, it is still not clear whether constitutively activated NFκB plays a causal role in tumour formation. The mechanisms of NFκB activation in mammary tumours are also not totally understood. It is easy

for the cyclin D1 gene, as a common marker for BC, to be activated by NFκB activation in mammary tumorigenesis because the NFκB binding site was found in the cyclin D1 promoter, and *in vitro* experiments demonstrated its activation by NFκB (Guttridge *et al*., 1999; Hinz *et al*., 1999). Furthermore, cyclin D1 overexpressed in the mammary gland can increase the incidence of mammary carcinomas, and knock out of cyclin D1 gene protects mice against BCs induced by MMTV-neu or MMTV-v-Ha-ras (Wang *et al*., 1994; Yu *et al*., 2001). It has been proved that ErbB2 and Ha-Ras can trigger signalling cascades that lead to NFκB activation during their involvement in cellular transformation *in vitro*. Results of studying the mice cell lines which carry MMTV-driven neu (ErbB2), heregulin/NDF, TGFα , v-Ha-ras, and c-myc transgenes have shown that certain oncogenes require and activate specific signal transduction pathways, including ERK/MAP kinase, JNK/SAP kinase, PI3K/AKT kinase, protein kinase C or the Src-related kinase pathways (Finco *et al*., 1997; Jo *et al*., 2000; Pianetti *et al*., 2001; Zhou *et al*., 2000).

NFκB is a cellular marker of an exciting molecular target. It is clear that NFκB activation, associated with the promotion of cell growth in mammary tumours and the inhibition of NFκB, possibly increases apoptosis and thereby increases BC response to chemotherapy and radiation. Future strategies that inhibit NFκB activation may prove to be of therapeutic value.

There is a need for clinical studies analysing NFκB as a predictor of response to treatment in BC.

1.4.4 NFκB and cancer therapy

NFκB plays an important role in many physiological processes, including innate and adaptive immune responses, cell proliferation, cell death, and inflammation. It has been widely accepted that regulation of NFκB activity in cell signalling pathways is not only involved in cancer development and progression, but also in resistance to chemo- and radiotherapy. Here, I will focus on the relationship between NFκB activity and cancer chemoresistance.

Although NFκB can either promote or oppose tumour development, first and foremost, NFκB plays a major anti-apoptotic role in established tumours and in tumour metastases (Baldwin *et al*., 2001). Substantial evidence indicates that aberrant sustained activation of NFκB has been reported in numerous tumours, and was implicated in various stages of tumourigenesis (Greten *et al*., 2004; Baldwin *et al*., 2001). As a negative regulator of NFκB, the tumour-suppressor gene CYLD binds to IKKγ, inhibiting the phosphorylation and degradation of IκB and thereby maintains NFκB in the cytoplasm (Brummelkamp *et al*., 2003). Inhibition of CYLD enhances activation of NFκB and its loss promotes cancer

development in families with familial cylindromatosis (Brummelkamp *et al*., 2003). Furthermore, NFκB regulates expression of cell-adhesion molecules that are associated with tumour metastasis (Bonizzi *et al*., 2004). It has been reported that expression of IκBα in tumour cells decreases the frequency of metastases, which indicates that the NFκB pathway can promote metastasis (Huang *et al*., 2000).

Nowadays, many chemotherapeutic agents have been discovered and successfully applied in treating patients with different types of cancer, such as BC. However, chemoresistance becomes the major obstacle of the cytotoxic effects of anti-cancer drug to effective cancer treatment. NFκB is activated in response to treatment with cytotoxic drugs. The NFκB pathway impinges on many aspects of cell growth and apoptosis. It has been reported that in *HeLa* cells, the topoisomerase I inhibitor SN38 (7-ethyl-10-hydroxycamptothecin) and the topoisomerase II inhibitor Dox both induce NFκB nuclear translocation. The activation of NFκB targets genes directly through mobilization and stimulation of the IKK complex which is leading to cell survival (Bottero *et al*., 2001). Another study of vinblastine showed that the enhancer activity of the NFκB-binding site is upregulated by vinblastine thereby leading to the activation and nuclear translocation of NFκB. These results demonstrated that microtubule disassembly induced by vinblastine can transactivate the nuclear proto-oncogene MYC through

NFκB, which will induce cell proliferation (Bourgarel *et al*., 2001). Interestingly, the upregulation of TNFα by chemotherapeutic agents induces not only apoptosis through a FAS-associated death domain/caspase dependent pathway, but also a survival pathway through the activation of AKT, which can activate IKK, leading to NFκB nuclear translocation. All these results indicated that anticancer drugs simultaneously activate several pathways that positively and negatively regulate the cell death process, and the modulation of the balance between death and survival signals plays a critical role in the process of cell apoptosis or proliferation.

It has been reported that constitutive NFκB activation is a reason for tumour cells escaping apoptosis, which is also related to the development of drug resistance in cancer cells (Patel *et al*., 2000; Arlt *et al*., 2001; Mabuchi *et al*., 2004; Kikuchi *et al*., 2003). Constitutive NFκB activation is involved in genetic alterations, which affect the genes encoding NFκB or IκB. It has been reported that a similar trend has been detected between overexpression of p65 at the mRNA and protein level and the expression of the anti-apoptotic FLICE-inhibitory protein (FLIP) in 5-fluorouracil (5-FU)-resistant colorectal and BC cell lines. The 5-FU-resistant cells showed high NFκB DNA-binding activity and co-transfected p65 and p50 cDNA induced 5-FU resistance in MCF7 BC cell lines (Wang *et al*., 2004). It has been believed that the most important function of p53 tumour suppress

gene is its ability to induce apoptosis, and disruption of this process can promote tumour progression and chemoresistance (Tergaonkar *et al*., 2002). Activated NFκB inhibits chemotherapy-induced stabilization and activation of p53, which results in resistance to chemotherapy (Tergaonkar *et al*., 2002; Webster *et al*., 1999). NFκB promotes cell survival through expression of genes encoding anti-apoptotic proteins or directly blocking caspase activation. The expression of BCL-XL and BFL1 play an important role in the survival effect of NFκB in response to chemotherapeutic drugs in human lung carcinoma cell line (Cheng *et al*., 2000). In addition, recent studies have demonstrated that NFκB has a consensus-binding site for the human *MDR1* (Bentires-Alj *et al*., 2003). This finding is further supporting the important role for NFκB in chemoresistance. All these data indicate that NFκB is a crucial target for cancer therapy and future cancer prevention.

NFκB is known to inhibit apoptosis through induction of anti-apoptotic proteins and/or suppression of pro-apoptotic genes, thus understanding the mechanism of apoptosis and the role of NFκB in apoptosis will contribute to the discovery of a new target for cancer therapy.

1.4.5 NFκB and hypoxia

Aerobic organisms of human body need adequate oxygen for maintaining life. Decrease in O_2 availability will trigger a wide range of processes to

adapt to these changed conditions. Hypoxia is characterized by a decreased O_2 tension within cells and can occur under several physiologic and pathophysiological situations such as cancer and inflammation. Hypoxia response can be divided into different time scales, including an acute, an intermediate and a chronic response, and in different levels of oxygen concentration, including a moderate (5-8% O_2) and an anoxic level (<1% O_2) (normoxia is 21% O_2) (Brahimi *et al*., 2007; Toescu *et al*., 2004; Lee *et al*., 2007). Hypoxia activates a number of genes that are important in the cellular adaptation to low oxygen conditions. Hypoxia inducible factor (HIF) is considered as a key regulator of hypoxia-inducible genes implicated in many different cellular functions such as cell survival, cell proliferation, apoptosis, glucose metabolism, and angiogenesis. HIF, also known as ARNT (aryl hydrocarbon nuclear translocator), is a heterodimeric factor composed from a tightly regulated α-subunit by PHDs (prolyl-hydroxylases) and constitutively expressed β-subunit (Gordan *et al*., 2008). HIF is primarily regulated at the protein expression level in hypoxia through hydroxylation-dependent targeting to ubiquitination and subsequent proteaosomal degradation.

There are three members in HIF family, HIF1, HIF2 and HIF3. HIF1 is ubiquitously expressed, whereas HIF2 is only expressed in endothelial cells and in the kidney, heart, lungs and small intestine (Semenza *et al*., 2001;

Piret *et al*., 2002; Gordan *et al*., 2008). HIF1α and HIF2α are closely related, and both activate hypoxia-response elements (HRE)-dependent gene transcription whereas HIF3α is the more distantly related isoform (Wenger *et al*., 2002). However, it has been reported that HIF1α and HIF2α may have distinct transcriptional targets (Rankin *et al*., 2007). Distinct roles for HIF1α versus HIF2α in promoting tumour growth have been most clearly defined in von Hippel–Lindau (VHL) disease–associated clear cell renal carcinoma (CCRC) (Raval *et al*., 2005). Furthermore, other data show the support of distinct roles for HIF1α and HIF2α in regulating cell differentiation and in promoting the growth of certain tumours (Kaelin *et al*., 2002; Covello *et al*., 2005). The HIF1 is composed of HIF1α and HIF1β subunit, both of them belong to the basic helix-loop-helix and PAS domain proteins family (Bracken *et al*., 2003). Under normoxic conditions, oxygen-sensitive HIF1α is degraded rapidly by hydroxylation of specific prolyl residues (Pro 402 and Pro 564), which are catalyzed by PHDs domain-containing proteins (Schofield *et al*., 2004). Furthermore, the VHL tumour suppressor protein promotes further degradation of HIF1α through the ubiquitin-proteasome pathway in this process (Maxwell *et al*., 1999). Under hypoxic conditions, HIF1α is stabilized, hypoxia triggers HIF1α to translocate into the nucleus binding with HIF1β, and transactivates downstream target genes containing HRE within their promoter or enhancer.

More than 50 years ago, it was first reported that human tumours grew under hypoxic condition. Hypoxia contributes to cancer cells resistance to apoptosis and mediates resistance to chemotherapy and radiotherapy (Thomlinson *et al*., 1955; Brown *et al*., 1998). Nowadays, hypoxia in human cancers, such as BC, the head and neck and cervical, is associated with increased metastasis and poor survival in patients (Hockel *et al*., 1999; Hockel *et al*., 2001). Nowadays, it is well accepted that HIFs play a pivotal role in tumour progression and metastasis. High expression of HIF1α, HIF2α and HIF3α proteins have been found in human cancers, which is associated with tumourigenesis and patient mortality (Kim *et al*., 2004; Semenza *et al*., 2003; Maynard *et al*., 2007). Furthermore, a few studies indicate that HIF1 may mediate resistance to chemotherapy and radiation (Unruh *et al*., 2003). Therefore, further investigation of the HIF1 pathway and understanding the regulation of its transcriptional activity may contribute to the discovery of a new therapeutic target for cancer treatment. Currently, several approved therapeutic agents such as Trastuzumab (Herceptin) and Camptothecin have been reported which inhibit HIF1 activity either by inhibition of the ability of HIF1 to interact with proteins, or through inhibition of signal transduction pathways (Blau *et al*., 2001). However, none of these drugs specifically target HIF1 even if they inhibit HIF1 activity. The lack of specific targeting increases the difficulty in

making clinical responses in patients, but these drugs still have a potential as anticancer agents (Kim *et al*., 2004).

HIF1α stabilization and induction was shown to be NFκB dependent under normoxic conditions (Bonello *et al*., 2007). HIF1α is continuously produced in the cells but quickly degraded under normoxic conditions by tanscriptional and post-transcriptional regulation. Increasing the expression and transcription rate of HIF1α is involved in activation of protein kinase C and NFκB. Reactive oxygen species (ROS) induce activation of the PI3K and AKT pathway increases the translational rate of HIF1α protein (Semenza *et al*., 2003). The stabilization of HIF1α is also implicated by inflammatory cytokines, transforming growth factor B, lipopolysaccharide, and stem cell factor in various cell types (Pedersen *et al*. 2008). For a long time, HIF1α was the major factor investigated in tumour hypoxia, but recently it has been demonstrated that active HIF2α can contribute to the development of tumour aggressiveness. HIF2α becomes stabilized at higher oxygen tensions than HIF1α in both non-malignant and malignant cells. High HIF2α expression has been linked to poor prognosis in several tumour types (Bonello *et al*., 2007). Therefore, our data showed that only HIF2α (but not HIF1α) plays a critical role in BC cell lines and BC stem cell related study.

A number of studies have now shown that cross-talk exists between the NFκB and HIF signalling pathways (Fig. 1.6). NFκB stimulates the transcription of HIF1, and HIF1 was shown to regulate the activation of NFκB (Scortegagna *et al*., 2008). The human HIF1α promoter contains NFκB binding site at a site -197/-188 base pairs upstream of the transcriptional start site, the mutation of which leads to a loss of hypoxic HIF1α up-regulation. All NFκB subunits could activate the HIF1α promoter which is supported by evidence that NFκB family members bind to the HIF1α promoter. *In vitro*, inflammatory mediators increase HIF1α mRNA expression through NFκB dependent mechanisms. *In vivo* study demonstrated that NFκB up-regulates HIF1α through transcriptional up-regulation and furthermore that basal NFκB is a prerequisite for constitutive HIF1α expression (Rius *et al*., 2008). Their study showed NFκB has a pivotal role in the high level of constitutive HIF1α mRNA. On the other hand, HIF1α can induce NFκB activation. Firstly, phosphorylation of IκB leads to its degradation releasing p65 for nuclear translocation. p65 was then phosphorylated on Ser276 by ERK1/2. In general, inhibition of NFκB might influence the activity of HIF1α and vice versa. Further investigation of the mechanisms of hypoxic activation of HIF and NFκB and how these signalling pathways interact will guide a new therapeutic strategy.

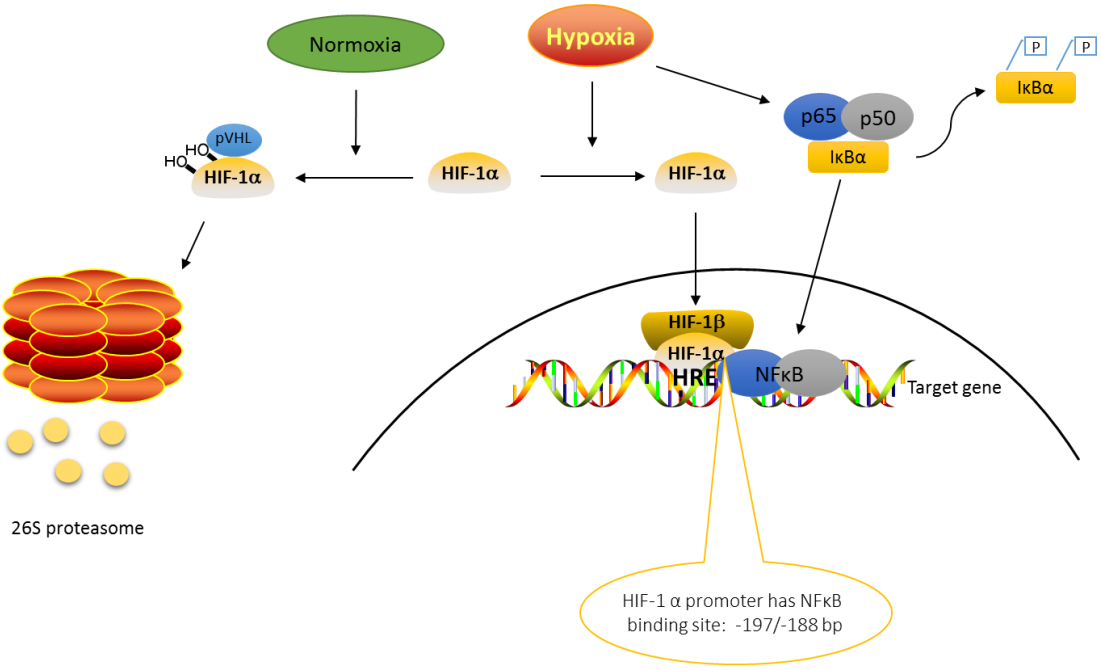


Fig. 1.6 Cross-talk between HIF and NFκB in hypoxia. Under normoxic conditions, activated HIF1α binds to pVHL and is followed by proteasomal degradation. Hypoxia leads to phosphorylation of IκB, releasing NFκB to the nucleus, where it binds to a distinct element at 197/188 bp of the HIF1α promoter, thus increasing HIF1α mRNA and protein levels. (Adapted from Scortegagna *et al*., 2008)

1.5 Cancer stem cell

All tissues in the body have a small population of organ-specific stem cells that are defined by their capacity to perpetuate themselves through self-renewal as well as to differentiate into the mature cells that comprise each organ. There has been a growing interest recent years in the role stem cells play in health and disease.

1.5.1 The concept of cancer stem cells

It has been proposed that stem cell biology could provide new insights into cancer biology. The relationship between stem cells and tumour cells is developed from the following observation. First, normal stem cells and cancer cells share the similar mechanisms that regulate self-renewal; second, tumour cells might be derived from normal stem cells; and third, the notion of CSCs with unclear proliferative potential that drives the differentiation and growth of tumour cells.

During the past century, many scientists tried to develop a theory to explain cancer pathogenesis and resistance to medications, but the controversial topic still exists and there is a largely unknown field in the cancer pathogenesis. In a series of landmark experiments, Jacob Furth and Morton Kahn demonstrated that after transplantion into a mouse, a single leukaemic cell was able to produce a new hematopoietic malignancy (Furth *et al*., 1937). This means certain cells from the cancer cell population might possess the ability to repopulate the cancer tissue and initiate new tumour growth. In 1960, McCulloch and other researchers found evidence to demonstrate that a small population of cells can renew themselves after injecting bone marrow cells into irradiated mice (McCulloch *et al*., 1960). All these experiments establish the princple of CSC and provide support for the CSC hypotheses.

The early definition of the CSC concept is described as “a concept of neoplasms”, based upon developmental and oncological principles. It states that carcinomas are caricatures of tissue renewal, in that they are composed of a mixture of malignant stem cells, which have a marked capacity for proliferation and a limited capacity for differentiation under normal homeostatic conditions, and of the differentiated, possibly benign, progeny of these malignant cells (Pierce *et al*., 1988). With a greater understanding of the role which mutations in oncogenes play in cancer, a clonal evolution concept of CSCs was proposed by Nowell, who described that “most neoplasms arise from a single cell of origin, and tumour progression results from acquired genetic variability within the original clone allowing sequential selection of more aggressive sublines. Tumour cell populations are apparently more genetically unstable than normal cells. The acquired genetic instability and associated selection process, most readily recognized cytogenetically, results in advanced human malignancies being highly individual karyotypically and biologically. Hence, each person’s cancer may require individual specific therapy, and even this may be thwarted by emergence of a genetically variant subline resistant to the treatment” (Nowell *et al*., 1976). More recently, with accumulating data from CSCs research, the new CSCs hypothesis has been proposed in line with the other concepts. This hypothesis has two separate but related components. First, tumours originate in either tissue stem cells or their immediate progeny

through dysregulation of the normally tightly regulated process of self-renewal. Second, tumours contain a group of cells that have key stem cell properties, which include self-renewal and differentiation.

Although some scientists have questioned whether CSCs are genuine or not, the existence of CSCs has been confirmed by a number of studies on experimental animal models. The first confirmation that CSCs existence was reported by the John Dick group in 1994. They successfully identified and purified human acute myeloid leukaemia initiating cells with distinct stem properties (Lapidot *et al*., 1994). After this, CSCs have been well identified and isolated in various hematological as well as solid malignancies. In recent years, detecting specific surface markers and signalling pathways in CSCs is the main research strategy that leads to development of new drugs targeting CSCs in the future. There are several limitations and inconsistencies regarding the explanation of CSC origin, CSC identification and isolation in CSC hypotheses. Therefore, many authors thought that the CSC model couldn't on its own explain as the only reason of cancer pathogenesis. There are many connections between stem cells and cancer that are important to understand. More research on stem cell biology is producing insight into the origins of cancer and will ultimately yield new approaches to fight this disease. The promise of more effective new cancer therapies has proven a powerful driver of CSC

research, and several theories, supported by sound and sophisticated experimental evidence, now merge into a more consistent picture.

1.5.2 Role of cancer stem cells in tumourigenesis

The current understanding of human cancer is that cancers can be caused by the accumulation of specific genetic mutations. Tumourigenesis involves aberrant tissue organization and cell proliferation. CSC is the key point to link these two events together. First, CSC provides a cellular mechanism that can explain how the genetic and epigenetic changes give rise to the tissue changes, and these mechanisms may get involved in cancer initiation, progression, invasion, and metastasis. It has been reported that overpopulation of mutant colonic SCs is the key point between initiating genetic changes with the earliest tissue changes in colon tumourigenesis in hereditary cancer patients (Boman *et al*., 2001; Boman *et al*., 2004). This report indicates that cancer initiation involves the development of CSC overpopulation. A number of recent papers show that many cancers contain a small population (~1%) of CSCs (Al-Hajj *et al*., 2003; Ginestier *et al*., 2007; Singh *et al*., 2004; O'Brien *et al*., 2007). However, these cells can regenerate up to 10^8 - 10^{13} in total number of fully developed cancer cells. Indeed, this must have occurred during all phases of tumourigenesis (Boman *et al*., 2007). It has been found that dysregulation of crypt mechanisms can lead to initiation and progression of

colon cancer. Moreover, SC overpopulation links abnormalities at the gene level and abnormalities at the tissue level from cancer initiation to metastasis (Boman *et al*., 2008). There is a growing amount of evidence, which suggests that metastases develop when distant organs are seeded with CSCs that arise from a primary tumour. These facts indicate that CSCs play a critical role in the seeding and growth of metastatic lesions (Balic *et al*., 2006).

1.5.3 Cancer stem cells marker

In the early nineties, some crucial technologies were developed for studying hematopoietic stem cells and bone marrow transplantation. A single CSC was identified in acute myeloid leukemia (AML) by FACS detecting the cell surface markers $CD34^{+}CD38^{-}$ and mouse xenograft assays (Lapidot *et al*., 1994; Bonnet *et al*., 1997). Nowadays, an important step in characterizing CSCs is the detection of specific surface markers. This may impact future strategies in anti-cancer drugs design and development. Nevertheless, further research efforts are required to determine the clinical significance of these findings, and to show whether CSCs are a universal property of all cancers. CSCs have been successfully isolated from many types of cancers, including the breast, brain, blood (leukemia), skin (melanoma), head and neck, thyroid, cervix, lung, organs of the gastrointestinal and reproductive tract and retina (Mimeault *et al*.,

2007). Although many CSC-specific surface markers have been identified (Table.1.5), the targeted therapeutic destruction of CSCs remains a challenge. Here, I will mainly discuss some important CSCs markers, which I am going to use in my study.

Table 1.5 CSC markers for distinct solid tumour types

Breast	Glioma	Colon	Liver	Lung	Melanoma	Ovarian	Prostate	Pancreatic
ALDH1	CD15	CD24	CD13	ABCG2	ABCB5	CD24	ALDH1	ABCG2
CD24	CD90	CD26	CD24	ALDH1	ALDH1	CD44	CD44	ALDH1
CD44	CD133	CD29	CD44	CD90	CD20	CD117	CD133	CD24
CD133	Nestin	CD44	CD90	CD117	CD133	CD133	CD166	CD44
CD90		CD133	CD133	CD133	CD271		Trop2	CD133
ESA		CD166						C-Met
		LGR5						CXCR4
		ABCB5						Nestin
		ALDH1						Nodal-Activin

(Medema *et al*., 2013)

As shown in the table above, although different types of cancer may have specific CSC markers, for example, CD271 in melanoma stem cells (Quintana *et al*., 2010), there are a few "pan" CSC markers that are expressed in a range of distinct malignancies. These markers are not only highly relevant for studies on the biology of CSC, but also could serve as useful markers to monitor the efficacy of differentiation therapy and may have a potential role as a targeted in cancer therapy.

Aldehyde dehydrogenase (ALDH) is a functional marker of CSCs (Ginestier *et al*., 2007). High ALDH activity in the cells may lead to cells resistant to chemotherapy drugs (Ginestier *et al*., 2007). There are 17 isoforms of ALDH in the human body that also localize to the mitochondria in addition to cytosol (Sladek *et al*., 2003). The kidney and liver have been observed to express the highest levels of ALDH (Lissa *et al*., 2013). ALDH has been extensively considered to be a potential marker for CSCs due to ALDH activity, which has been linked to normal multipotent stem and progenitor cells (Lissa *et al*., 2013). ALDH positive cell population appeared to have better engraftment potential in NOD/SCID mice than their ALDH negative counterparts in acute myeloid leukemic cells (Cheung *et al*., 2007). ALDEFLUOR flow showed that on average 8% of normal mammary epithelial cells had ALDH activity (Ginestier *et al*., 2007). Furthermore, ALDH positive BC cells were capable of forming xenograft tumours with as few as 500 cells. However, ALDH negative cells cannot form xenograft tumours with as many as 50,000 cells. When we isolate breast CSCs by ALDH combined with $CD44^{+}/CD24^{-}$ markers, as few as 20 positive cells could form tumours. ALDH activity as a marker for CSCs can be used in a number of other solid tumours such as lung, pancreas, prostate, liver and head and neck squamous cancer (Ucar *et al*., 2009; Jimeno *et al*., 2009; Hellsten *et al*., 2011; Ma *et al*., 2008; Clay *et al*., 2010).

As early as 1984, the potential role of ALDH in chemoresistance had already been identified in a cyclophosphamide-resistant L1210 leukemic cell line by John Hilton (Hilton *et al*., 1984). An experiment result regarding early passage colon cancer xenograft tumours found that more ESA^+, $CD44^+$, ALDH positive cell populations were in the surviving tumour cells after cyclophosphamide treatment. When these ESA^+CD44^+ colon CSCs have treated with ALDH inhibitors or ALDH1A1-targeted siRNA, CSCs sensitivity to cyclophosphamide increased (Dylla *et al*., 2008). That means the chemoresistance seen in their model was specifically attributed to elevated ALDH activity (Dylla *et al*., 2008). Continued studies with ALDH inhibitors or inhibitors related pathways that influence ALDH activity may provide useful tools in overcoming chemoresistance in CSCs or directly impairing CSC growth.

The other prominent markers among these are CD34, CD133, CD44, and CD24 (Al-Hajj *et al*., 2003; Singh *et al*., 2003; Ginestier *et al*., 2007). CD34 is one of the first CSC markers to be well characterized in acute myeloid leukemia stem cells as a hematopoietic stem cell marker (Singh *et al*., 2004). As a famous CSC surface marker, CD133 has also been widely investigated since its identification as a marker of glioblastoma multiforme stem cells (You *et al*., 2010). CD133 is a glycosylated protein with five transmembrane domains and two large extracellular loops. At the

beginning, CD133 positive phenotype was used to identify and isolate brain tumour stem cells in malignant tumours, but more recently, it has been used to define the CSC populations in some different types of cancer, including breast, lung, pancreatic, liver, prostate, gastric, colorectal, and head and neck cancers. CD133 positive cell populations show the ability to maintain CSCs in multiple tissues. Furthermore, CD133 positive cells undergo multi-lineage differentiation to neurons, astrocytes, and oligodendrocytes *in vitro*. *Nestin*, *BMI1*, *Olig2*, and *Nanog* genes have been found up-regulated in CD133 positive populations of brain, lung, liver and prostate cancers (Ma *et al*., 2008; Miki *et al*., 2007; Bertolini *et al*., 2009). CSCs are considered to be one of the reasons for resistance to traditional chemotherapies. CD133 positive cells have an increased survival rate *in vitro* and have been enriched *in vivo* after treatment with cisplatin (CDDP), etoposide, Dox, and PAC (Chen *et al*., 2008; Zhang *et al*., 2010a). CD44 is an 85-90kDa transmembrane glycoprotein containing 10 standard exons and four major domains, including the hyaluronan binding and variably spliced regions, the transmembrane sequence and the intracellular cytoskeletal-signalling domain (Fang *et al*., 2012). CD44 is presenting the characteristics of CSCs across tissues, including breast, pancreas, gastric, head and neck, ovarian and colon, whereas CD24 is not (Verkaik *et al*., 1999). Results showed that invasive CD44 positive prostate cells also induced the expression of Nanog, BMI1 and SHH, which is similar to

CD133 positive cells (Klarmann *et al*., 2009). $CD44^{+}$and $CD24^{-}$ is a marker to define the breast CSCs population (Al-Hajj *et al*., 2003). Around 50% of tumour initiating cells with a wide spectrum of CSC markers could be found in this whole tumour cell population, such as CD133, CD271 and ALDH1. Despite the huge number of melanoma stem cells, some have argued that the CSC model may not apply to melanoma (Quintana *et al*., 2010; Roesch *et al*., 2005). The rate of CSC population can vary widely from 1% to 50% based on the different cancer type with their tumorigenic potential (Hoek *et al*., 2010). Moreover, other studies have suggested that the ability of CSCs to transition between invasive and proliferative states is governed by prevailing tumour microenvironment (Hoek *et al*., 2010). Regardless of the debate over the existence of CSCs, tumour transdifferentiation still presents an attractive therapeutic opportunity because the cancer cells will transit to a postmitotic and terminally differentiated state, which could significantly attenuate tumour progression.

1.5.4 Cancer stem cells and chemotherapy

Nowadays, although significant advances have been made in molecular biology and clinical medicine, cancer still remains a major public health problem all over the world. Standard chemotherapy and radiotherapy have failured to eliminate all malignant cells in the body. Chemotherapy remains an important part of treatment for most types of cancer. Unfortunately, 90%

of drug failures in metastatic cancers are attributed to chemoresistance leading to cancer recurrence (Longley *et al*., 2005). Understanding the mechanisms of chemoresistance is very important for developing new therapeutic approaches to treating cancer. There is growing evidence that CSCs play a critical role in chemoresistance in a number of cancers. Identification and isolation of CSCs in various tumours is the first step to investigating the mechanisms by which CSCs can contribute to tumour initiation as well as continued tumour progression. Understanding the mechanisms of CSCs in chemotherapy and tumour relapse is important as it provides clues to better addressing cancer therapy.

It is often suggested that CSCs are resistant to therapy in the same way that normal stem cells are protected against insult. These protection mechanisms include, quiescence (Guzman *et al*., 2002), expression of ABC drug pumps (Jones *et al*., 2004), high expression of anti-apoptotic proteins and resistance to DNA damage (Richardson *et al*., 2004). They may display increased resistance to chemotherapy agents compared with more differentiated cells that comprise the bulk of tumours. It has been reported that leukemic stem cells are more resistant to chemotherapy than are the more differentiated myeloblastic cells that constitute the vast majority of cells in leukemia. A similar finding has shown that myeloma stem cells are resistant to the chemotherapy used for myeloma therapeutics (Guzman *et*

al., 2002; Jones *et al*., 2004; Richardson *et al*., 2004). Long-term quiescence may be a crucial mechanism for the resistance of CSCs to anti-proliferative chemotherapy. CSCs occur in a quiescence state under certain conditions. Cell cycle assay shows that many CSCs are not cycling and are in G0 and thus resistant to cell cycle-specific chemotherapy agents. Moreover, stem cells are often identified by their propensity to retain DNA labels much longer than their rapidly proliferating offspring. This would explain the appearance of local recurrence or distant metastasis after long lag periods. Apart from this, there are still several molecular mechanisms that may lead to the resistance of CSCs to apoptosis.

1.5.5 Cancer stem cells and hypoxia

Over the past decade, accumulating data indicated that hypoxia contributes to cancer progression by activating adaptive transcriptional programs that promote cell survival, motility and tumour angiogenesis. I have discussed the role of NFκB in the cancer cells under hypoxic condition and the interaction between NFκB and HIF pathways in the previous paragraphs. Hypoxia can activate the NFκB signalling pathway in cancer cells. NFκB stimulates the transcription of HIF-1, and HIF1 was shown to regulate the activation of NFκB (Scortegagna *et al*., 2008). In general, inhibition of NFκB might influence the activity of HIFs and vice versa. CSCs are rare in cancer cells but have been recognized as a new mechanism to explain

chemoresistance and tumour relapse. Here, I will discuss hypoxia and CSCs concepts, the molecular connections between hypoxia-induced transcription factors (HIFs) and NFκB in CSCs, and how these results add an important new facet to our traditional view of hypoxia and cancer.

Stem cell "niche" is defined as a physiologically limited and specialized microenvironment that maintains the combined properties of stem cell self-renewal and multipotency. The common features and functions of all stem cell niches have been described as follows. (a) The stem cell niche is composed of a special population of cells that functions to maintain stem cells. (b) Stem cells are anchored in the niche by adhesion molecules, which are involved in the interaction between stem cells and the niche and between stem cells and the extracellular matrix. (c) The niche generates extrinsic factors that control stem cell numbers through the balance between proliferation and differentiation. (d) Hypoxia is a major characteristic of cells in the niche. Both normal stem cell and CSC share the common features of self-renewal and slow cycling in the niches to protect them from chemotherapy. The CSCs in the hypoxic niche have been implicated in tumorigenesis (Simsek *et al.*, 2010).

CSCs are slow growing subpopulations of tumours with the ability to repopulate eradicated cancer cells resulting in cancer recurrence and patient mortality. Most solid tumours exhibit hypoxic conditions inside their mass.

Hypoxia is both a physiologic stimulus and a micro environmental feature in a range of cancers including BC and brain tumours. Hypoxia may lead to increased resistance to radiotherapy and chemotherapy through activation of HIF factors in cancer therapy. Moreover, hypoxia may directly change the function of tumour cells with stimulation of de-differentiate and release angiogenic factors to increase blood and oxygen supply (Holmquist *et al*., 2006). It is believed that CSCs play critical roles in treatment resistance and recurrence after conventional therapy through multiple mechanisms and networks. A number of studies have confirmed that hypoxia and HIF signalling pathway play an important role in both intrinsic and acquired resistance to conventional therapeutics in most solid tumours. Clonogenic assay showed that high expression of HIF1 increased the resistance to radiation under both normoxic and hypoxic conditions. Meanwhile, when the activation of HIF1 signalling pathway has been blocked by HIF1α inhibitors, the sensitivity of tumour to radiation therapy has increased *in vitro* and *in vivo* (Liao *et al*., 2007; Strofer *et al*., 2011). Several mechanisms of how hypoxia and HIF pathway lead to therapeutic resistance have been proposed, some of which are similar to the CSCs resistant mechanisms, such as hypoxia-induced cell cycle arrest, impaired DNA repair system, altered cellular metabolisms through multiple signalling pathways, and decreased cytotoxicity due to the lack of oxidation of DNA free radicals by hypoxia. It has also been considered that hypoxia

induced development and maintenance of CSCs would contribute to increased therapeutic resistance.

There is plenty of supportive evidence showed that hypoxia and HIF signalling pathway play pivotal roles in the regulation of the phenotype and function of CSCs. Hypoxia regulates the sub-population of normal stem cells and maintains the normal tissues or non-stem cell tissues in a stem cell state during embryonic and adult development (Fraker *et al*., 2009; Dunwoodie *et al*., 2009). In glioma CSCs, hypoxia enhances the self-renewal capacity and maintenance of undifferentiated state of CSCs (Soeda *et al*., 2009; Panchision *et al*., 2009). Hypoxia maintains the stem-like phenotypes in neuroblastomas and activates signalling pathways that are associated with undifferentiated phenotypes of normal stem cells (McCord *et al*., 2009). Moreover, hypoxia triggers cancer stemness genes such like (sex determining region Y)-box 2 (Sox2), octamer-binding transcription factor 4 (Oct4) and Nanog may be one of the major reasons of tumour aggressiveness. Accumulating data show that there is a special areas which is hypoxic called niche in a tumour and it is also the areas of necrotic tumour tissues where CSCs reside (Simsek *et al*., 2010). It is possible that tumours may develop from mutation in normal stem cells or from non-stem cell population during hypoxic conditions. It is believed that HIF1α and HIF2α are the main factors to regulate the hypoxia-mediated phenotype and

function of CSCs (Zeng *et al*., 2011; Heddleston *et al*., 2010). The expression of both HIF1α and HIF2α are associated with tumour aggressiveness. Several studies have demonstrated that hypoxia-induced HIF-1α overexpression promotes the phenotype and function of CSCs, and up-regulation HIF targeting genes such as Oct4, Nanog, c-Myc, Notch-1, and CD133 (Li *et al*., 2010; Mathieu *et al*., 2011; Covello *et al*., 2006; Gordan *et al*., 2008). HIF2α also plays an important role in the regulation of CSCs. In my study, it has been shown that HIF2α is highly expressed in CSCs of BC. Hypoxia induces the expression of HIF2α thereby increasing the expression of stem cell markers including CD133, Oct4, Nanog, Sox2, and c-Myc in CSCs (Bar *et al*., 2011). All these findings strongly suggest that HIF1α and HIF2α are required for the phenotype and function of CSCs.

HIFs activate the expression of at least 150 genes encoding proteins, including NFκB. The NFκB is a well-known critical transcription factor in a wide range of cancers that regulates the expression of NFκB target genes associated with a variety of cellular processes. Moreover, NFκB also plays an important role in CSCs, contributing to tumour aggressiveness (Wang *et al*., 2009; Sarkar *et al*., 2008; Widera *et al*., 2008). Many studies have indicated that hypoxia could activate NFκB signalling pathway in cancer cells. NFκB is primarily a regulator of anti-apoptotic gene expression. It is very possible to believe that under hypoxic condition, the activation of

NFκB is required to inhibit apoptosis to enable a hypoxic cell to survive. Both HIF and NFκB signalling pathways are play a critical role in maintenance of stem cell phenotype and function in tumour microenvironment. Overall, it is reasonable to speculate that hypoxia induced NFκB activation may be contributing to the maintenance of CSCs during the development and progression of tumours. Understanding of the precise molecular mechanism of hypoxia regulation of NFκB in CSCs would allow designing of targeted approach for the development of new therapeutics to improve the overall survival of malignancies patients.

1.6 Disulfiram

Disulfiram (DS), tetraethylthiuram disulfide, was developed in 1881. A German chemist called M. Grodzki had synthesized a new compound from thiocarbamide with its stoichiometric formula $C_{10}H_{20}N_2S_4$ (Grodzki *et al*., 1881). Twenty years later, DS was first introduced in the developing rubber industry to accelerate the vulcanization of rubber (Eneanya *et al*, 1981) and widely used in the vulcanization of both natural rubber and synthetic rubber products. From the early 1940s, DS was used in medicine by two British physicians as it was a promising drug against scabies (Gordon *et al*., 1942). Then, it was used in the treatment of chronic alcoholism based on the finding of Hald and Jacobsen that ingestion of alcohol after DS treatment

would lead to highly disagreeable reactions (Eneanyn *et al*, 1981). Its clinical use over a long period of time has shown it to be a very safe drug with minimal and manageable toxic effects, even at fairly high doses of 300 to 500 milligrams a day. Its bioavailability is excellent (more than 80%), and after taking the drug 20% remains in the body for up to two weeks (Sauna *et al*., 2005).

After cases of alcoholic BC patients being treated with DS whose cancer spontaneously regressed, some scientists began investigations into the possible uses of DS in cancer treatment (Wang *et al*., 2003). As cancer research further develops, a key issue in anticancer therapy is reversing drug resistance. Many investigations have been carried out into agents that may target more than one cellular pathway in order to decrease the development of drug resistant tumour cells or to sensitise cancer cells to chemotherapy drugs (Guo *et al*., 2009; Wang *et al*., 2003).

1.6.1 Disulfiram metabolism

DS could be viewed as two molecules of diethyldithiocarbamic acid molecules joined together by a disulphide bridge with its molecular weight 296.54 (Fig. 1.7).

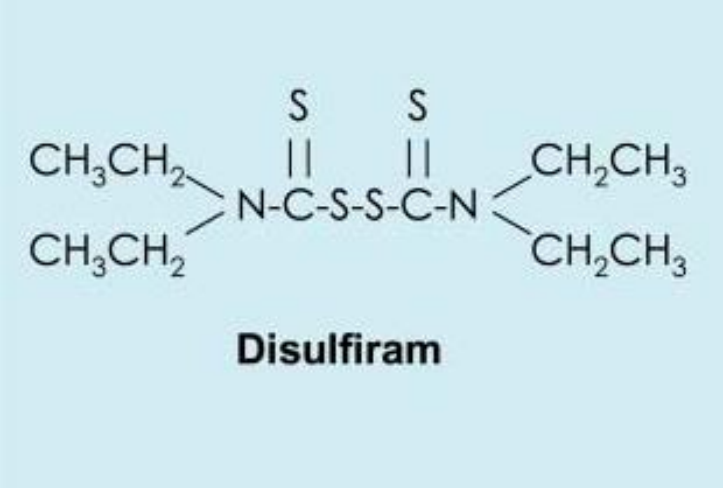


Fig. 1.7 Chemical structure of Disulfiram (Sauna *et al*., 2005)

The metabolism of DS is described in Fig. 1.8. First, DS (I) is converted to diethyldithiocarbamic acid (DDC) (II) by a reduction of the disulfide linkage to its corresponding thiol. Some groups reported that the glutathione reductase can reduce DS in the erythrocytes. The data showed that up to 50g of DS can be decreased by the adult human erythrocyte within 24 hours (Stromme *et al*., 1963). *In vitro* studies showed that DS was promptly reduced to the thiol within 4 min (Cobby *et al*., 1977). There are four different pathways for DDC to be further metabolized, including glucuronidation, nonenzymatic degradation, methylation, and oxidation.

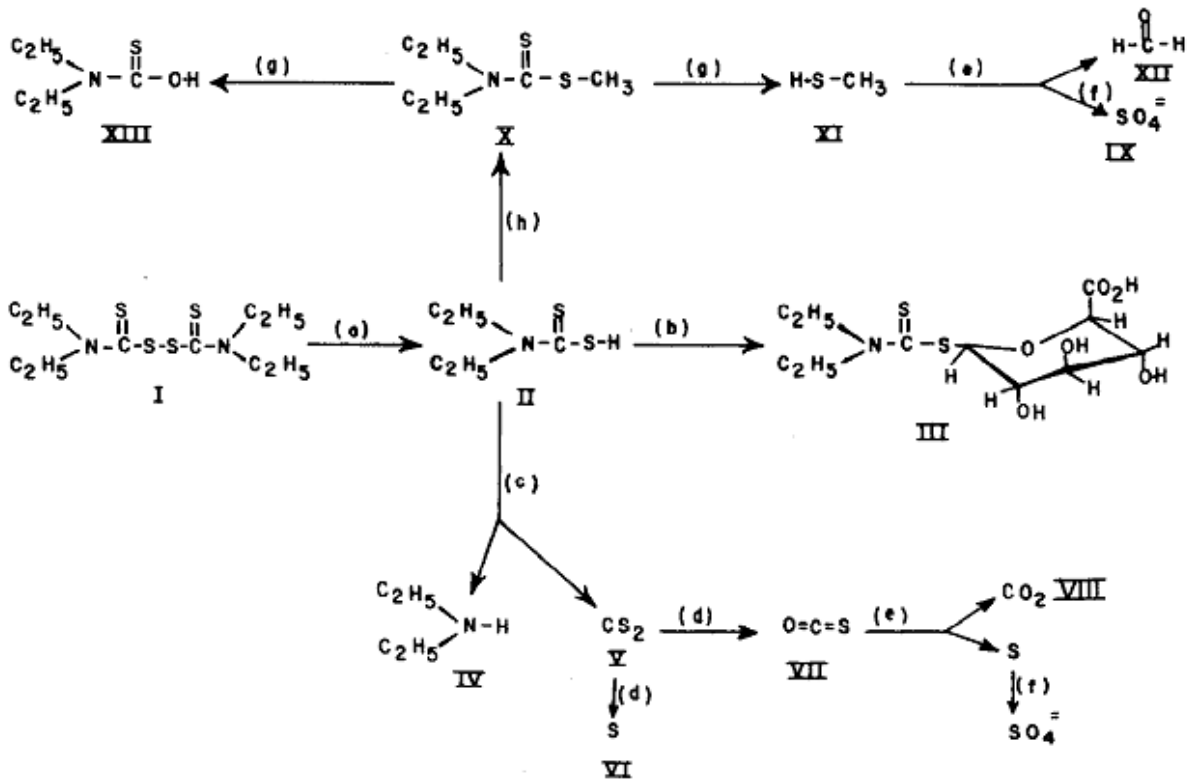


Fig. 1.8 The metabolic fate of disulfiram. I. Tetraethylthiuram disulfide (disulfiram), II. Diethyldithiocarbamic acid, III. Glucuronide of diethyldithiocarbamate, IV. Diethylamine, V. carbonyl disulfide, VI. Sulfur, VII. Carbonyl sulfide, VIII. Carbon dioxide, IX. Sulfate, X. methyl ester of diethyldithiocarbamate, XI. Methyl mercaptan, XII. Formaldehyde, XIII. thiocarboxylic acid. (a) Glutathione reductase, (b) conjugation, (c) nonenzymatic degradation, (d) oxidative desulfuration ($C\text{-}P_{450}$), (e) oxidation, (f) sulfoxidase, (g) esterases, (h) S-methylation: S-adenosyl methionine transmethylase. (Eneanyn et al, 1981)

Glucuronidation The main detoxification mechanism of DS is DDC conjugation of glucuronic acid in mammals (Fig. 1.8). 50% of a given dose of glucuronide metabolite is excreted in urine by the metabolism studies of radioactive DS in a rat. (III) (Stromme *et al*., 1966) (Fig. 1.9). However, there is still another possibility, that a small number of the conjugated

glucuronide are hydrolyzed by the esterases in the intestine during enterohepatic circulation without relating to DDC.

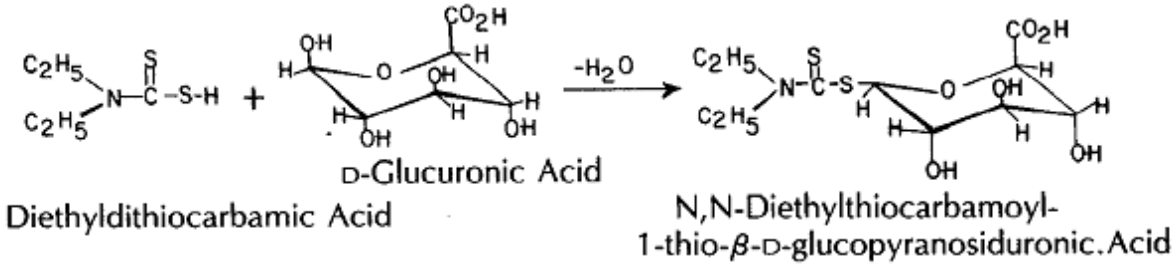


Fig. 1.9 Glucuronidation of diethyldithiocarbamic acid (Eneanyn et al, 1981).

Nonenzymatic degradation DDC decomposition rate is pH dependent. DDC decomposes rapidly to diethylamine (IV) and carbon disulphide (V) in acid environment (Stromme *et al*., 1966). There is a possibility that diethylamine may be further degraded to ammonia and to acetaldehyde. Carbon disulfide is oxidatively desulfurated to carbonyl sulfide (VII) and sulfur with the help of NADPH and cytochrome P_{450} (Dematteis *et al*., 1974). Carbonyl sulfide can be further oxidized to carbon dioxide and sulfur, meanwhile, it can be metabolized to sulfate by the sulfoxidases (Faiman *et al*., 1978). During either carbon disulfide poisoning or DS intoxication, a large number of sulfate ions is found in the urine of mammals from these observations

Methylation Radiochemical experiments results showed that there is still 0.05% of the given dose of DS present as methyl ester of DDC (MeDDC) intraperitoneally and 2 hours later (Cobby *et al*., 1977). Furthermore, when

DDC was administered to dogs intravenously, S-methylation accounted for approximately 27% of metabolism of the given dose (Gessner *et al*., 1972). S-methylation is normally implicated in the metabolism thiopurines, thiopyrimidines, and thiopentals (McBain *et al*., 1969). Esterases may target the methyl ester of the DDC derivative (X) to generate a methyl mercaptan which may in turn be oxidized to sulfate and formaldehyde. It is important to know that the production of sulfate from other thioester compounds is a rapid process. For example, 6-methylmercaptopurine generates sulfates quicker than 6-mercaptopudne when administered to rats (Canellakis *et al*., 1953). The possible reason may be S-methyl compound oxidation to produce inorganic sulfate by cleavage of methyl mercaptan (Maw *et al*., 1954).

This methylation pathway is an important sulfate-forming pathway in the metabolism of DS as 62-74% of sulfate formed, which is involved in methylmercaptan oxidation (Gessner, *et al*., 1972). The methyl ester of DDC does not go through the covalent disulfide interchange reaction by which DS inhibits aldehyde dehydrogenase, so it is very stable in blood (Kitson *et al*., 1976). Thioalcohol (XIII) formed from this methylester can be glucuronidated and excreted in urine.

Oxidation It is known that under atmospheric oxygen condition, DDC can be reoxidized to DS. Interesting data demonstrated that DDC can change

back to DS by reoxidation (Strume *et al*., 1965), but only about 4% of DDC involved. Such a reoxidation can be effected by the oxidases in the body.

1.6.2 Disulfiram and diseases treatment

1.6.2.1 Anti-alcoholism

DS has been used for over 50 years as an anti-alcoholism drug (Petersen *et al*., 1992; Johnsen *et al*, 1992; Kampman *et al*., 2009). DS and many of its metabolites (S-Methyl-N,N-diethylthiocarbamate sulfoxide (MeDTC-SO), S-Methyl-N,N-diethyldithiocarbamate sulfoxide (MeDDC-SO) and S-Methyl-N,N-diethyldithiocarbamate sulfine (MeDDC-sulfine)) are irreversible inhibitors of the enzyme ALDH1 by oxidising essential sulfhydryl groups to form irreversible internal S-S bonds (Vallari *et al*, 1982). Alcohol consumption after having taken DS would lead to the build-up of acetaldehyde in the plasma as a result of alcohol oxidation. ALDH1 plays a role in the removal of acetaldehyde. Acetaldehyde in the plasma would mediate histamine release leading to flushing of the skin, owing to cutaneous vasodilatation particularly on the trunk, hypotension because of decreased diastolic blood pressure, reflex tachycardia, tachypnoea, a sensation of warmth, palpitations, anxiety, headache, nausea, and vomiting.

1.6.2.2 Anti-cancer treatment

DS has also been studied as a potential anti-cancer drug (Brar *et al*, 2004). *In vitro*, DS demonstrated the ability to inhibit the growth of several cancer cell lines. Importantly, in a single case study on a 64 year old woman with hepatic metastasis that originated from ocular melanoma, DS showed the ability to suppress hepatic metastasis and shrink the tumours by >50% in size. In addition, the woman remained clinically well and physically active after 53 continuous months of DS therapy (Brar *et al*, 2004). It has been reported that DS could sensitise chemotherapy resistant tumours to chemotherapy treatments by inhibiting ATP-driven efflux pumps in cancer cells (Sauna *et al*, 2005). On top of chemosensitizing effect of DS, studies also showed that DS is cytotoxic to cancer cells in a biphasic manner (Wickstrom *et al*, 2007; Cohen *et al*, 1990). A clinical trial has shown that the prognosis was improved for a group of BC patients who received anticancer treatment plus DS compared with another group who had the same treatment without DS (Dufour *et al*., 1993). To date, although the full effects of DS are still not fully understood, studies have shown that DS and its metabolites target a number of different cellular pathways within the human body and have several mechanisms of action, mainly due to their metal chelating properties and reactions with key cysteine residues in particular proteins (Sauna *et al*., 2005). These signalling pathways include,

inhibition of DNA topoisomerase preventing DNA transcription and replication, inhibition of MMPs leading to the inhibition of tumour cell metastasis, inhibition of the proteasome leading to inhibition of the NFκB signalling pathway and downstream expression of cancer survival genes, and inhibition of superoxide dismutase (SOD) and increasing intracellular copper (Cu) level leading to toxic build-up of ROS (Sauna *et al*., 2005).

1.6.2.3 Reactive Oxygen Species and Copper

ROS are essential for biological functions. The unpaired electrons of oxygen react to form partially reduced highly reactive species that are defined as ROS, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical, and peroxynitrite. Mitochondria are considered the major source of cellular ROS, electron leakage from the mitochondrial respiratory chain may react with molecular oxygen, resulting in the formation of superoxide, which can subsequently be converted to other ROS (Schafer *et al*., 2001). ROS can be also gained through a reaction catalysed by NADPH oxidase complexes or as a by-product of certain biochemical reactions, such as β-oxidation in peroxisomes, prostaglandin synthesis and detoxification reactions by cytochrome P450 (Perry *et al*., 2000). A mild increase in the level of ROS can promote cell proliferation and differentiation, whereas excessive amounts of ROS can cause irreversible oxidative damage to lipids, proteins and DNA, leading to cell death

(Boonstra *et al*., 2004; Schafer *et al*., 2001; Perry *et al*., 2000). Therefore, maintaining the ROS level is important for cell growth and survival. The ROS level is controlled in cells by balancing ROS generation with their elimination by ROS-scavenging systems such as superoxide dismutases (SOD1, SOD2, and SOD3), glutathione peroxidase, peroxiredoxins, glutaredoxin, thioredoxin and catalase (Boonstra *et al*., 2004). Cancer cells are known to be metabolically active and under increased oxidative stress. Increased cellular ROS regulates signalling pathways by directly reacting with and modifying the structure of proteins, transcription factors and genes to control cell growth and differentiation. The following mechanisms are considered to contribute to increase ROS in cancer cells. (a) Oncogenic signals inducing ROS generation. (b) Malfunction of the mitochondrial respiratory chain leading to increased amounts of ROS. (c) High level of ATP energy demand putting a further stress on the mitochondrial respiration chain to induce ROS generation. (d) Decreasing the expression or the activity of antioxidant enzymes, which causes ROS accumulation (Davies *et al*., 1999).

High ROS stress can induce various biological responses, including adaptation, increased cell proliferation, DNA damage, apoptosis, and necrosis (Davies *et al*., 1999). In order to protect cells against increased ROS stress, one adaptation mechanism is the glutathione system

(GSSG/2GSH) and thioredoxin system, which maintain cellular redox balance (Schafer *et al*., 2001; Sasada *et al*., 1996). Another mechanism is to up-regulate the expression of antioxidant enzymes such as SOD, catalase, and peroxidases. It is known that an increase of certain ROS such as superoxide and hydrogen peroxide may promote the proliferation of cancer cells due to modulation by ROS of the redox states of signalling molecules. The transcription factors NFκB, HIF-1α and intermediate signalling molecules such as MAPK and JNK have also been shown to be involved in ROS-mediated modulation of cell growth and cell survival (Martindale *et al*., 2002). Moreover, ROS could induce DNA lesion due to its capacity to attack various components of DNA, leading to the generation of a variety of ROS-mediated modified products including oxidized bases, DNA strand breaks, and DNA intra-strand adducts (Randerath *et al*., 1996; Lloyd *et al*., 1997). Excessive ROS may induce cellular injury and cell death by damaging the mitochondrial membrane, and this damage is likely to cause the release of cytochrome c and activate the apoptotic cascades (Hensley *et al*., 2000). ROS show low or mild level in many cancer cells, and further increased ROS will lead cells to apoptosis after exposure to anti-cancer drugs.

Cu is an essential trace element for humans and high intracellular concentrations of Cu have been found to generate ROS in cells which can

damage DNA, protein and lipid membranes eventually resulting in apoptosis. The transport of Cu into cells is tightly controlled by a member of the Cu transporting family which termed Ctr1. As DS is a strong metal chelator (Morrison *et al*., 2010), it can increase the cellular uptake of Cu leading to activation of ROS and apoptosis. ROS also activates NFκB, triggering anti-apoptotic factors which decrease this effect but the DS/Cu complex both increases ROS and inhibits NFκB, so the overall effect seems to sensitise cells to chemotherapy drugs (Guo *et al*., 2009; Liu *et al*., 2012; Yip *et al*., 2011). It has been reported that DS can enhance the anti-tumour efficacy of external-beam γ-irradiation and ^{131}I-metaiodobenzylguanidine (^{131}I-MIBG) through induction of ROS and inhibition of proteasome activity (Rae *et al*., 2013). Moreover, DS used alone or in combination with Cu, causes loss of mitochondrial membrane potential, and activates the production of ROS and death caspases in malignant haematological cell lines (Conticello *et al*., 2012). The important role of ROS in this was shown as the effect could be reversed by addition of the antioxidant N-acetylcysteine. There have also been reports of DS, in conjunction with zinc, triggering ROS production leading to apoptosis in pancreatic adenocarcinoma cell lines. The apoptotic effects of DS/zinc were stronger when used in combination with dFdC, and the effects were even more pronounced in dFdC-resistant cells (Dalla *et al*., 2011).

Due to the high oxygen demands of cancer cells and the inability of the microenvironment to deliver this demand, cancer cells develop hypoxia (Ruan *et al*, 2009). Under hypoxia conditions, cancer cells increase the production of ROS as a result of incomplete metabolism. ROS are signalling molecules that activate the expression of and stabling of hypoxia-inducible factors (HIFs). HIFs are transcription factors that activate hypoxia response genes, such as VEGF and SOD (Ruan *et al*, 2009; Tuller *et al*, 2009).VEGF promotes blood vessel growth (angiogenesis) to the tumour, thus providing the tumour with its own blood supply of unlimited source of nutrients and a route for tumour metastasis (Chen *et al*., 2009). In addition, ROS also inhibit PTEN, a phosphatase which inhibits AKT signalling pathway by the dephosphorylation of phosphatidylinositol-3, 4, 5-trisphosphate (PI-3, 4, 5-P3) to phosphatidyl-4, 5-bisphosphate (PI-4, 5-P2) (Fruehauf *et al*, 2007). Activation of the AKT pathway would inhibit apoptosis and stimulate cell proliferation. However, too much ROS is cytotoxic and could damage nuclear and mitochondrial DNA. Therefore, ROS levels are tightly controlled in cancer cells by maintaining an adequate level of SOD and glutathione-peroxidase. SOD catalyses the conversion of ROS to H_2O_2 and H_2O_2 is converted to water by glutathione-peroxidase (Fruehauf *et al*, 2007). Interestingly, high Cu levels would induce hepatocellular injury and cell death. Cu-induced cell death is associated with an increase in ROS generation ($O2^-$) through reduction of

Cu^{2+} to Cu^{1+} in the GSH to GSSH. This would eventually deplete the glutathione pool leading to accumulation of high ROS levels and cell death. Cancer cells carefully maintain this balance of Cu and ROS levels to prevent death while accessing the ROS signalling pathway to stimulate hypoxia-induced cancer cell proliferation. DS has been shown to increase the intracellular Cu level (Cen *et al*, 2004).

1.6.2.4 Cytotoxic mechanisms of disulfiram

The exact mechanisms of DS to target cancer cells still remains to be elucidated. However, studies suggested that DS targets many signalling pathways which are essential for the survival of cancer cells. *In vivo* and *in vitro* studies showed DS to be an effective anti-cancer drug capable of reducing cancer cell proliferation and metastasis. More importantly, the cytotoxicity of DS is cancer specific (Daniel *et al*., 2005). The theory has been put forward that the mode of cancer specificity is due to elevated intracellular Cu level in cancer cells. DS chelated Cu to form a complex $Cu(deDTC)_2$inside cancer cells (Margalioth *et al*., 1983). This would lead to the inhibition of the proteasome and NFκB pathway. The DS/Cu complex is the active inhibitor of the proteasome, therefore, the higher Cu level in cancer cells would result in higher DS/Cu complex formation, which is toxic to cancer cells. The level of DS/Cu complex formed in

normal cells does not reach the toxicity level observed in cancer cells, thus making DS-cytotoxicity cancer-specific.

Inhibition of aldehyde dehydrogenase and NFκB

It has been found that amplified ALDH activity can be used as a marker of CSCs (Ginestier *et al*., 2007) and can be a factor in causing resistance of BC cell lines MDA-MB-231 and MDA-MB-468 to chemotherapeutic drugs and radiotherapy (Croker *et al*., 2012). DS inhibits ALDHs (Eneanya *et al*., 1981) and studies from our groups found that that DS/Cu inhibited the ALDH-positive cells in a BC stem cell population, preventing them from creating mammospheres after 24 hours of exposure to the drug.

NFκB is a transcription factor and the signalling pathways which activate it have a central role in the immune system's response to infection. It is found in the cytoplasm of normal cells as an inactive protein complex consisting of five subunits, p50/p105, p52/p100, p65(RelA), RelB and C-Rel which can bind as homo- or hetero-dimers to the promoter region of target DNA sites (known as κB sites) to trigger gene expression downstream in the signalling pathway. When stimulated, its inhibitor IκB is phosphorylated, ubiquitinised and degraded, therefore allowing NFκB dimers to be freed from the complex, which then translocate to the nucleus to begin transcription. Therefore, NFκB plays an important role in cancer development and progression. Inhibition of the proteasome is an indirect

method of inhibiting NFκB. Because NFκB is constitutively active and over-expressed in cancer cells, it suggests that NFκB must be vital for cancer cell survival, development and metastasis. Therefore, inhibition of NFκB would result in the inhibition of cancer cell survival, development and metastasis.

It has been shown that DS and its active metabolites such as diethydithiocarbamate (DDC) can inhibit NFκB activity, which has been activated by ROS, in colorectal cancer cell lines, p53 mutant DLD-1 and p53 wild-type RKOWT, by interacting with the sulfhydryl groups on the Rel homology (RH) domain, which enhances the cytotoxic effect of 5-FU anticancer drug and reverses the resistance of the cell line to 5-FU (Wang *et al*., 2003). Our groups showed that a DS and Cu complex increased the cytotoxicity of dFdC by inhibiting the increased NFκB activity caused by over-exposure to the drug and reversed the resistance to the drug in breast and colon cancer cell lines. NFκB activity has been shown to be decreased by DS in hepatoma Hep G2 cells, and this study also showed that DS caused apoptosis and G1/S arrest mediated by p53 (Liu *et al*., 1998). Other research demonstrated that when treated with DS, the NFκB activity decreased in cerebral endothelial cells, in this case thought to be due to its ability to increase cellular levels of zinc (Kim *et al*., 2000). Data showed that induction of cleaved caspase-3 (a marker of apoptosis) expression

occurred via inhibition of the NFκB signalling pathway when human herpes virus-8 infected primary effusion lymphoma cells were treated with DDC (Matsuno *et al*., 2012).

1.7 Project aims

CSCs play a main role in cancer cell chemoresistance which is the major obstacle for the success of BC chemotherapy. Therefore, development of CSCs-targeting drug is of significant clinical importance. The ultimate goal of my study is to determine the anticancer efficacy of a newly developed liposome encapsulated disulfiram (Lipo-DS) *in vitro* and *in vivo*. This project was designed to achieve the following aims.

To determine the relationship between hypoxia and CSCs and investigate the function of NFκB in bridging hypoxia and CSC-related chemoresistance in BC cell lines.

To investigate the CSC traits and chemoresistant characteristics in acquired chemoresistant BC cell line.

To examine the cytotoxic effect of a newly developed Lipo-DS on BC cell lines, especially BCSCs, in vitro and *in vivo.*

To investigate the molecular anticancer mechanisms of Lipo-DS *in vitro* and *in vivo*.

2. Material and Methods

2.1 Materials

2.1.1 General reagents, enzymes and kits

Enhanced Chemiluminescence Western Blot Signal Detection Kit (Amersham Biosciences, UK)

BD FACSCalibur™ Flow Cytometer, CellQuest™ Pro (BD Biosciences, Oxford, UK)

Stuart® 3 block heater (Bibby Scientific Ltd., Straffordshire, UK)

EZ-ECL chemiluminescence detection kit for horse radish peroxidise Solution A and B (Biological Industries Beit Haemek Ltd., Isreal)

Bio-Rad protein assay kit II (Bio-Rad Laboratories, Hertfordshire, UK)

Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L glucose, Foetal Calf Serum (FCS), L-Glutamate (200 mM) (BioWhittaker® Lonza, Walkersville, USA)

Penicillin-streptomycin mixture (10,000U/ml penicillin and 10,000U/ml Streptomycin) (BioWhittaker® Lonza, Walkersville, USA)

Sterile Phosphate Buffer Saline (PBS) (0.0067M PO4) (BioWhittaker® Lonza, Walkersville, USA)

Trypsin-EDTA (10x) (BioWhittaker® Lonza, Walkersville, USA)

Microscope slide (ERIE SCIENTICFIC Company Portmouth.N.H., US)

Fuji Medical X-Ray Film Super RX (13x18) (Fujifilm UK Ltd., Bedfordshire, UK)

Amersham Hybond ™ - P (PVDF membrane), Amersham Hybond ™ - N^+ (GE Healthcare, Buckinghamshire, UK)

30% acrylamide:bis-acrylamide (37.5:1) (GeneFlow, UK)

10× Transfer buffer (GeneFlow, UK)

10× Electricpresis buffer (GeneFlow, UK)

Semi-dry transfer unit (Hoefer, Inc., Holliston, USA)

NuPAGE ® LDS Sample Buffer (4x), SeeBlue plus 2 pre-stained protein markers (Invitrogen Ltd., Paisley, UK)

10×TAE buffer, G418, Trizol, Optimem, Hygromycin B, Lipofectamine transfection reagent (Invitrogen Ltd., Paisley, UK)

Nalgene Cryoware™ Cryogenic vials (Labware, Roskilde, Denmark)

Marvel dried milk (Marvel, Dublin, Ireland)

10, 25, 50 and 100 ml tubes (Merck Sharp & Dohme (MDS) Ltd., Hertfordshire, UK)

AccuGel 40% (19:1 acrylamide:bis), Running buffer (10X, contains distilled H_2O, Tris/Glycine/SDS) (National Diagnostics, Yorkshire, UK)

BamH I and Hind III enzymes and associated buffers (New England Biolabs)

Restriction enzymes and associated buffers, pfu polymerase, Luciferase assay kit, Gel shifting assay kit, 1Kb DNA ladder, 100Kb DNA ladder, Dual Luciferase Assay reagents, Access RT-PCR system (Promega UK Ltd., Southampton, UK)

Qiaquik gel extraction kit, Qiafilter maxiprep kit, Qiafilter miniprep kit (Qiagen, West Sussex, UK)

RNase A (100 mg/ml) (Qiagen Ltd., West Sussex, UK)

Complete EDTA free protease inhibitor tablets, Rapid Ligation kit (Roche Diagnostics Ltd., East Sussex, UK)

Complete easy packs-Protease inhibitor cocktail tablets (Roche Diagnostics Ltd., West Sussex, UK)

96-well flat-bottom tissue culture plates with lids, Tissue culture Cell+ flasks with PE ventilation caps, Filtropur V50.0.2 Vacuum Filter (500 ml), Cell scrapper (Sarstedt Ltd., Leicester, UK)

disulfiram, Cu Chloride ($CuCl_2$), minisapt plus (0.20 μm) filter, cisplatin (CDDP), paclitaxel (PAC) , doxorubicin (Dox) , gemcitabine (dFdC), 99.9% Dimethylsulfoxide (DMSO), Tris HCl, Tris Base, Tween 20, EDTA, Ammonium persulfate (APS), Sodium Dodecyl Sulfate (SDS, 98.5% GC grade), Methanol, Isopropanol, Glycine, HEPES, Sodium Chloride (NaCl), Thymidine, KH_2PO4, Sodium hydroxide (NAOH), Nonident p40, Ethidium Bromide, LB broth, polydIdC (PdIdC), Triton X-100, Agarose, 3MM paper, Propidium iodide (powder), Tetramethylethylenediamine (TEMED), fixer/replenisher, developer/replenisher, DL-dithiothreitol (DTT) solution (1 M in H_2O) (Sigma Aldrich Company Ltd., Dorset, UK)

Multiskan Ascent and Multidrop 384 (Thermo Fisher Scientific Inc., Leicestershire, UK)

2.1.2 Antibodies

p-p65 (S276) antibody (Abcam, Cambridge, UK)

Horse radish peroxidase conjugated secondary anti-mouse and anti-goat antibodies (Amersham Biosciences, UK)

Ki-67 antibody (BD Biosciences, Oxford, UK)

AKT, p-AKT, Sox2, Oct4, Nanog, JNK, p-JNK, c-Jun, p-c-Jun, p-p38, ERK antibodies (Cell Signalling, Herts, UK)

Enhanced ChemiLuminescence (ECL) ™ anti-mouse antibody (Ab), ECL ™ anti-rabbit antibody (GE Healthcare, Buckinghamshire, UK)

HIF2α antibody (Novus Biologicals, CO, USA)

p65, dCK, RRM1, p21, BCL-2, BAX, Cleaved PARP, Nucleolin, ALDH1A1, ALDH2, ALDH1A3, IκBα, MDR1 antibodies (Santa Cruz Biotechnology Inc., Heidelberg, Germany)

Anti-vinculin monoclonal antibody, Anti-tubulin monoclonal antibody, FITC-conjugated anti-mouse IgG antibody （Sigma Aldrich Company Ltd., Dorset, UK）

2.1.3 Cell lines

Human breast cancer cell lines: MCF7, T47D, ZR75, and MDA-MB-231

Human normal breast epithelial cell line: MCF10A （American Tissue Culture Collection, Rockville, USA）

The normal human vascular endothelial cells, HeCV （Prof Wenguo Jiang, University of Cardiff, UK）

Human endothelial cell line: EAhy926 （Dr Angel Armesilla, University of Wolverhampton, UK）

NFκB p65 transfected (P1, C2) and mock transfected MCF7 human breast cancer cell line （Dr Weiguang Wang, University of Wolverhampton, UK）

2.1.4 Buffers

Complete protease inhibitor

A (25x) complete protease inhibitor (Roche Diagnostics Limited, West Sussex, UK) was prepared by dissolving 1 tablet of complete protease inhibitor in 25 ml of distilled water, and stored at -20 ℃ until use.

Glycine Buffer

Glycine buffer solution (500 ml) was prepared by dissolving 3.75 g glycine and 2.92 g NaCl in distilled water. The pH of the glycine buffer was adjusted to pH 10.5 using 5 M NaOH. The glycine buffer solution was stored in room temperature until use.

RIPA Buffer for Whole Protein Extraction

10x RIPA Buffer was prepared by the list below, and RIPA Buffer was stored in -20 ℃ until use.

Tris HCL	25 g/l
Sodium dodecyl sulphate	0.1% per ml
Triton-X 100	1%
NaCl	0.15 M
EDTA	1 mM
Protease inhibitor	0.001% per ml

Buffer A for Nuclear Protein Extraction

Buffer A was prepared by mixing 200 µl 500 mM HEPES, 50 µl 2 M KCl, 2 µl 500 mM EGTA (pH 8.0), 2 µl 500 mM EDTA (pH 8.0), 20 µl 0.5 M DTT, and 500 µl (25X) protease inhibitor in 9.2 ml water. Buffer A was stored in -20 ℃ until use.

Buffer C for Nuclear Protein Extraction

Buffer C was prepared by mixing 60 µl 500 mM HEPES, 120 µl 5M NaCl, 3 µl 500 mM EGTA, 3 µl 500 mM EDTA, 3 µl 0.5 M DTT, 75 µl (25X) protease inhibitor, and 75 µl 100% glycerol in 1161 µl water, and stored at -20 ℃.

Separating buffer

Separating buffer was prepared by mixing 90.8 g Tris base and 2g SDS in 500 ml of distilled water. The pH of the solution was then adjusted to pH 8.8 using HCl. The separating buffer was stored in room temperature until use.

Stacking buffer

Stacking buffer was prepared by mixing 30 g Tris base and 2 g SDS in 500 ml of distilled water. The pH of the solution was then adjusted to pH 6.8 using HCl. The stacking buffer was stored in room temperature until use.

Tris-buffered saline Tween-20 (TBS-T)

A stock (10x) TBS-T solution was prepared by dissolving 12.11 g Tris base and 81.8 g NaCl in 1 litre of distilled water. The (1x) TBS-T buffer was then prepared by mixing100 ml (10x) TBS-T, 900 ml distilled water and 500 µl of Tween-20.

Blocking buffer

Blocking buffer was prepared by dissolving 5 g Marvel milk powder in 100 ml of (1x) TBS-T.

Transfer buffer

Transfer buffer was prepared by mixing 100 ml of 10 x stock solution, 200 ml of methanol and 700 ml distilled water. This solution was stored at room temperature until use.

Running buffer

Running buffer was prepared by mixing 100 ml of 10X stock solution with 900 ml distilled water.

2.2 Methods

2.2.1 Cell culture

Serum-containing medium

Serum-containing medium consists of DMEM medium containing 1% (v/v) L-glutamate, 1% (v/v) penicillin-streptomycin and 10% Foetal Calf Serum (BioWhittaker® Lonza, Walkersville, USA)

Serum-free medium

Serum-free medium consists of DMEM medium containing 1% (v/v) L-glutamate, 1% (v/v) penicillin-streptomycin (BioWhittaker® Lonza, Walkersville, USA).

Trypsin

Working solution of trypsin (1x) was prepared by diluting stock solution of trypsin (10x) in sterile PBS (BioWhittaker® Lonza, Walkersville, USA)

Freezing Medium for cryogenic storage of cell samples

Freezing medium was prepared by mixing 90% foetal calf serum with 10% DMSO (Sigma Aldrich Company Ltd., Dorset, UK), and stored at 4 ℃ until use.

Recovering Cell Lines from Liquid Nitrogen Storage

A 75 cm^2 tissue culture flask (Sarstedt Ltd., Leicester, UK) was prepared by adding 19 ml of serum-containing medium. Cells were recovered from liquid nitrogen storage, rapidly defrosted in 37 ℃ water bath, and then transferring the 1 ml content of the cryovial into the prepared 75 cm^2 tissue culture flask. The recovered sample was then stored in an incubator, at 37 ℃ with 5% CO2.

Trypsinization of Adherent Cell Lines

Cell culture medium was removed from the tissue culture flask, rinsed with 5 ml of sterile PBS, and 2 ml of trypsin added to the adherent cells and spread evenly. The flask was then placed in the incubator and checked regularly, under the microscope and by gentle tapping of the flask, to

determine that the cells were no longer adhered to the tissue culture flask. When the cells were no longer adhered to the tissue culture flask, 2 ml of serum-containing medium was added and the cells were re-suspended thoroughly by pipetting the 4 ml volume up and down.

General Cell Line Maintenance

Cell cultures were regularly checked under the microscope (2-3 times a week) for cell density, and changes in the colour of the medium. When there is a change in the colour of the medium but the cell density is low, (defined as adhered cells are spread out and very few cells are touching each other), the medium is removed and replaced with fresh serum-containing medium. When there is a change in the colour of the medium and the cell density is high, (defined as cells are overgrown leading to some cells no longer being adhered to the tissue culture flask and are in suspension), cells are sub-cultured. Sub-culturing was done by trypsinizing the cells. The 4 ml of cells were then collected into a 15 ml falcon tube. In the case when cells were not needed, half of the cells (2 ml) were disposed of. In the case when cells were needed, the cells where then centrifuged at 800 g for 3 minutes. The medium was then removed and cell pellet was re-suspended in 2 ml of fresh serum-containing medium. Two 75 cm^2 tissue culture flasks were then prepared by adding 19 ml of serum-containing medium, and to this 1 ml of the cell suspension was then added. The cells

were then stored in an incubator, at 37℃ with 5% CO_2, with the tissue culture flask lying down.

Preparing Cell Lines for Liquid Nitrogen Storage

Cells were trypsinised, spun for 3minutes at 800 g and the cell pellet collected. The cell pellet was then re-suspended in freezing medium and aliquot into 1 ml/labelled-cryovial. Each cryovial was then wrapped in tissue paper, put into a disposable labelled-glove and placed in -80℃ overnight. The following day, the cryovials were removed from the gloves and tissue papers, and transferred into liquid nitrogen (-180℃) for long-term storage. The locations of the samples stored in liquid nitrogen are recorded in the liquid nitrogen storage log.

2.2.2 Cytotoxicity assay

Stock MTT solution 5 mg/ml MTT solution (500 ml) (Sigma Aldrich Company Ltd., Dorset, UK) was prepared by adding 2.5 g of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in 500 ml of sterile PBS, mixed to dissolve using a magnetic stirrer, with the bottle wrapped in foil to protect the MTT from direct light. This MTT mixture was then filtered using a vacuum filter connected to a pump. This filtered MTT solution, wrapped in foil, was stored at 4℃ until use.

MTT assay is one of the widely used methods to evaluate the effect of anti-cancer drugs on cell viability. Cells were cultured in 96-well plates (1×10^4/well) and left overnight to allow the cells attached to the bottom in the incubator at 37℃. Cells were exposed to different concentrations of anti-cancer drug prepared by a two-fold serial dilution using medium i.e. 10 μM, 5 μM, 2.5 μM, 1.25 μM, 0.625 μM, 0.3125 μM. Meanwhile, there was also a negative control of cells which was untreated. After removing the spent media from the wells, the medium (200 μl) with different concentrations of drug was distributed in each well. Normally there are 3 wells with the same drug concentration and cell type in each plate at the same time to calculate the standard deviation. After incubation at 37℃ for 72 hours, 50 μl of MTT (5 mg/ml) was added to the wells and the plates were wrapped in the aluminium foil to prevent the deterioration of MTT by light. The solution was removed after incubation for 4 hours at 37℃, and 60 μl of 99.9% DMSO and 10 μl glycine buffer were added. The readings were tabulated and IC50 of the cell lines was calculated from the graph after reading the plates by a spectrophotometer at a wavelength of 540 nm.

2.2.3 Western Blot

10% Ammonium Persulphate (APS)

10% Ammonium Persulfate (APS) (Sigma Aldrich Company Ltd., Dorset, UK) was prepared by dissolving 0.1 g APS in 1 ml of distilled water. This was prepared fresh before use.

Preparation of Separating Gel

Separating gel was prepared by adding 5.5 ml of separating buffer to 6.5 ml of distilled water. To this mixture, 4.4 ml of 40% (19:1) acrylamide:bis (AccuGel) (Sigma Aldrich Company Ltd., Dorset, UK) was added, followed by 120 µl of 10% APS and 15 µl TEMED (Sigma Aldrich Company Ltd., Dorset, UK). 4 ml of this separating gel solution was added to the glass gel rack. A small amount of isopropanol, just enough to cover the top of the separating gel solution, was added on top of the separating gel. The gel was left to set.

Preparation of Stacking Gel

Stacking gel was prepared by adding 4.2 ml of stacking buffer to 6.6 ml of distilled water. To this mixture, 1.2 ml of AccuGel was added. The isopropanol (Sigma Aldrich Company Ltd., Dorset, UK) from the separating gel was removed and rinsed with distilled water when the gel was set. Then to the stacking gel mix, 250 µl of 10% APS and 20 µl

TEMED was added. The final stacking gel mix was added on top of the separating gel. A comb was immediately placed on the top layer of the stacking gel, to form the protein loading wells, while avoiding bubbles. The gel was left to set before removing the comb.

Cytoplasmic and Nuclear Protein Extraction

Nuclear protein extracts were prepared by washing the cells in PBS, suspended in buffer A and left on ice for 15 minutes, to which, 5 µl 10% NP-40 (Sigma Aldrich Company Ltd., Dorset, UK) was added and then centrifuged for 5 minutes at 800 g. The supernatant was collected (cytoplasmic protein) and the pellet was re-suspended in 500 µl buffer A, centrifuged for 5 minutes at 800 g, and the supernatant removed. The pellet was then re-suspended in 100 µl of buffer C, left to spin on a rotator at 4 ℃ for 1 hour, then centrifuged for 5 minutes at 12,000 g and the supernatant collected (nuclear protein).

Protein Concentration Measurement

A BSA protein serial standard was prepared by serially diluting the 3.0 mg/ml BSA solution in RIPA buffer to the following concentrations: 3.0 mg/ml, 1.5 mg/ml, 1.0 mg/ml, 0.75 mg/ml, 0.50 mg/ml, 0.25 mg/ml and 0 mg/ml (30 µl RIPA buffer). The BioRad reagent A/S was prepared by adding 20 µl of reagent S to every ml of reagent A and mixed by vortex.

Protein samples were diluted to a 1:10 or 1:5 ratio in distilled water depending on the size of the cell pellet when preparing the protein sample. 5 µl of the BSA standards (duplicates) and the diluted protein samples were pipetted into a 96-well flat-bottomed plate, in triplicates, to which 25 µl of reagent A/S was added. 200 µl of reagent B was then added, incubated at room temperature for 10 minutes and then the absorbance read at 650 nm. The protein concentration of the samples was calculated from plotting the absorbance versus protein concentration of the BSA serial standard.

SDS-PAGE Electrophoresis

The prepared SDS-PAGE gels were assembled into the electrophoresis tank loaded with 1 x running buffer. The protein samples were prepared by mixing, in a 0.5 ml eppendorf, 16 µl of loading buffer (4x), 1 µl of 1 M DTT, 50 µg of protein sample and distilled water to make up a final volume of 64 µl. This mixture was then heated using a PCR block heater at 90 ℃ for 10 minutes, then centrifuged at 10,000 g for 30 seconds and stored on ice until use. 10 µl of pre-stained protein markers and 30 µl of each protein mixture were then loaded into the loading wells using a pipette. The gel was then run at 200 V for 1 hour.

Blotting

3MM paper (Sigma Aldrich Company Ltd., Dorset, UK) was cut into 12 x 12 cm size and wet with transfer buffer. PVDF membrane was cut into 7 x 9 cm size and wet with methanol. A semi-dry transferring unit was setup by placing the SDS-PAGE gel (Sigma Aldrich Company Ltd., Dorset, UK) on top of the PVDF membrane, which is then sandwiched by 2 layers of 3 x 3 MM paper. Protein was transferred from the SDS-PAGE gel to the PVDF membrane by apply 2 W current for 1 hour and 20 minutes.

Blocking of the membrane

The TBS-T(1x) working solution supplemented with 5% fat-free milk was used to block the protein-containing PVDF membrane (Sigma Aldrich Company Ltd., Dorset, UK) for 1 hour along with agitation in order to prevent non-specific binding of primary and secondary antibodies.

Antibody Staining

5 ml of primary antibody (Table 2.1) was prepared as described in the blocking buffer. After shaking the PVDF membrane in the blocking buffer for at least 1 hour, the membrane was placed in a plastic bag with the primary antibody, sealed, and left overnight at 4℃ on a shaker. On the following day, the PVDF membrane was washed twice with TBS-T (1x) for 10 minutes. The PVDF membrane was then soaked in the secondary

antibody at room temperature for at least 1 hour on a shaker. The PVDF membrane was then washed twice with TBS-T (1x) for 10 minutes.

Table 2.1 Primary Antibodies

Target Molecule	Dilution Ratio	Antibody Company
p-p65 (S276)	1:1000	Abcam, Cambridge, UK
Ki67	1:1000	BD Biosciences, Oxford, UK
AKT	1:1000	Cell Signalling, Herts, UK
p-AKT	1:1000	Cell Signalling, Herts, UK
Sox2	1:1000	Cell Signalling, Herts, UK
Oct4	1:1000	Cell Signalling, Herts, UK
Nanog	1:1000	Cell Signalling, Herts, UK
JNK	1:1000	Cell Signalling, Herts, UK
p-JNK	1:1000	Cell Signalling, Herts, UK
c-Jun	1:1000	Cell Signalling, Herts, UK
p-c-Jun	1:1000	Cell Signalling, Herts, UK
p-p38	1:1000	Cell Signalling, Herts, UK
ERK	1:1000	Cell Signalling, Herts, UK
HIF2α	1:1000	Novus Biologicals, CO, USA
HIF1α	1:1000	Novus Biologicals, CO, USA
p65	1:1000	Santa Cruz, Heidelberg, Germany
dCK	1:1000	Santa Cruz, Heidelberg, Germany
RRM1	1:1000	Santa Cruz, Heidelberg, Germany
p21	1:1000	Santa Cruz, Heidelberg, Germany
BCL-2	1:1000	Santa Cruz, Heidelberg, Germany
BAX	1:1000	Santa Cruz, Heidelberg, Germany
Cleaved PARP	1:1000	Santa Cruz, Heidelberg, Germany
Nucleolin	1:1000	Santa Cruz, Heidelberg, Germany
ALDH1A1	1:1000	Santa Cruz, Heidelberg, Germany
ALDH2	1:1000	Santa Cruz, Heidelberg, Germany
ALDH1A3	1:1000	Santa Cruz, Heidelberg, Germany
IκBα	1:1000	Santa Cruz, Heidelberg, Germany
MDR1	1:1000	Santa Cruz, Heidelberg, Germany
p53	1:1000	Santa Cruz, Heidelberg, Germany
Tubulin	1:8000	Sigma Aldrich, Dorset, UK

Signal detection

In the dark room, 2 ml prepared EZ-ECL solution (Thermo Fisher Scientific Inc., Leicestershire, UK) which was mixing solution A with solution B at a 1:1 ratio was added to PVDF membrane. Leave the PVDF membrane in the A/B mixture solution for 3-5 minute and then remove excess solution by pouring onto a tissue paper. The PVDF membrane was then wrapped in cling film and then exposed to a Fuji film. The length of exposure depends on the strength of the signal.

2.2.4 Flow Cytometry

Propidium Iodide

Propidium iodide (Thermo Fisher Scientific Inc., Leicestershire, UK) was prepared by dissolving 50 mg of propidium iodide powder into 50 ml of distilled water. This solution was kept at 4 ℃ wrapped in foil until use.

Preparation of Ethanol

70% ethanol (Thermo Fisher Scientific Inc., Leicestershire, UK) was prepared by diluting in PBS from ethanol stock solution.

After 72 hours incubation of cell cultures with the appropriate test drugs, the supernatant containing cells in suspension was collected into a 25 ml tube. In addition, adhered cells were trypsinised and also collected into the

same 25 ml tube. The cells were pelleted by centrifugation at 800 g for 5 minutes. The supernatant was removed and the cells washed twice with 5 ml of sterile PBS. The final cell pellet was re-suspended in 0.5 ml of sterile PBS. Using a vortex mixing the 0.5 ml cell suspension, 2 ml of 70% ethanol was slowly added and left for 10 minutes. After 10 minutes have passed, the cell was pelleted by centrifugation at 800 g for 5 minutes, the ethanol was removed and the cell pellet re-suspended in 1 ml of sterile PBS. To this cell suspension, 1 µl of 100 mg/ml RNaseA was added followed by 50 µl of 1 mg/ml propidium iodide and incubation for 30 minutes at room temperature wrapped in foil. Samples were stored at 4℃ until they were analysed by flow cytometry.

2.2.5 Electrophoretic mobility shift assay (EMSA)

Gel preparation

10 x TAE-10 ml of 50 x TAE + 40 ml of dH_2O (Invitrogen Ltd., Paisley, UK)

30% polyacrylamide Protogel, TEMED 10% Ammonium persulfate (APS)-1 g of APS + 1 ml of dH_2O (10% APS) (Thermo Fisher Scientific Inc., Leicestershire, UK)

Sample preparation

10 x Binding Buffer, 1 µg/µl Poly (dI-dC), 50% Glycerol, Biotin End-Labelled Target DNA, 5 x Loading Buffer (Thermo Fisher Scientific Inc., Leicestershire, UK)

Probes Preparation

Wild Type (WT) Unlabelled Target DNA (upper and downer probes) 40 µm. Mutant (MUT) Unlabelled Target DNA (upper and downer probes) 40 µm.

Annealing the upper and downer probes: The probes were heated at 100 ℃ for 10 minutes and were cooled down to room temperature. Store the samples at -20 ℃. Note: after the upper and downer probes have annealed, the final probe concentration is 20 µm since each annealed probe contains one upper and downer probe.

Samples Preparation

10 x Binding Buffer, 1 µg/µl Poly (dI-dC), 50% Glycerol, WT Unlabeled Target DNA, MUT Unlabeled Target DNA, and Biotin End-Labelled Target DNA were thawed on ice. Nuclear protein was added with final amount 25 nM of biotin-labeled oligonucleotide in binding buffer consisting of 10 mM Tris at pH 7.5, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM $MgCl_2$, and 50 ng of poly (dI-dC). WT and MUT samples

were prepared without adding Biotin End-Labelled Target DNA. The samples were incubated on ice for 15 minutes and then 10 minutes at RT. Meanwhile, the other samples were prepared without adding Biotin End-Labelled Target DNA. All samples were added 1 µl of Biotin End-Labelled Target DNA and were incubated on ice for 15 minutes followed by 10 minutes at RT. 5 µl of 5 x Loading Buffer were added into each sample.

Electrophoresis

All samples (23 µl/well) were loaded into their designated wells respectively, and then the gel was run for 45 minutes at 120 V on ice.

Blotting

The nylon membranes (Amersham Hybond ^{TM}-N^{+}, GE Healthcare, UK) and 3MM papers were soaked in 1 xTAE for a minimum of 5-10 minutes. The probes were transfered from the gel to the nylon membrane using wet or semi-dry units following manufacture protocol at 200 mA for 1 hour.

Cross-link

The membranes were wrapped in cling film and were exposed for 10-15 minutes on a trans-illuminator equipped with a 312 nm bulb (Ingenious Syngene Bio-Imaging).

Blocking

The blocking buffer and the 4 x wash buffer (Thermo Fisher Scientific Inc., Leicestershire, UK) were pre-warmed to 37-50℃ to dissolve any precipitation, then the membranes were soaked in blocking buffer for 15 minutes at room temperature on a shaker. At the same time, 30 µl Stabilized Streptavidin-Horseradish Peroxidase Conjugate were added to 10 ml Blocking Buffer. The membranes were soaked in the blocking buffer with conjugated HRP for 15 minutes at room temperature on a shaker.

Washing

1 x wash solution (40-60 ml) (Thermo Fisher Scientific Inc., Leicestershire, UK) were prepared for washing the membranes for 10 minutes twice in a clean tray, then the membranes were transferred to a new container adding 10 ml Equilibration Buffer for 5 minutes at room temperature on a shaker.

Signal detection

(Luminol/Enhancer Solution): (Stable Peroxide Solution) (Thermo Fisher Scientific Inc., Leicestershire, UK) were mixed in 1:1 ratio and were evenly spread on the membranes, for 5 minutes. The membranes were exposed to film in the dark room.

2.2.6 Luciferase reporter gene assay

Cell culture

Different cell lines were harvested by trypsinisation. Plate 5 x 10^4/100 µl of medium/well on to 96 well plates and allowed them to grow overnight at 37 ℃.

Lipofectamine Transfections

On the following day, the transfection solution I and II were prepared first, then the pGL3-Basic and other luciferase vectors (0.2 µg of each vector/well) in triplicate and internal control pRL-SV40 (0.002 µg/well) were co-transfected by using Lipofectamine 2000.

Solution I: 0.2 µg of each Luciferase reporter plasmid DNA and 0.002 µg pRL-SV40 (Renilla) DNA was diluted in 25 µl of opti-MEM medium without serum.

Solution II: 0.5 µl of lipofectamine 2000 was diluted in 250 µl of opti-MEM for each DNA samples and incubated at room temperature for 5 minutes.

The equal amount of solution I and solution II were mixed for 20 minutes at room temperature and then were added to the cell culture. The cells were

incubated at 37℃, 5% CO_2 for 24 hours and were fed with fresh medium for further 24 hours incubation prior to luciferase assay test.

Luciferase assay test & analysis

The growth medium was removed after 48 hours, and the wells were washed with 1 x PBS. 100 µl of 1 x lysate buffer was added to each well and incubated at room temperature for 10 minutes with agitation. 20 µl of each lysate sample was transferred into a polypropylene plate to test. 30 µl of Luciferase assay reagent II was added and read in a luminometer. The reading corresponds to firefly luciferase activity. 30 µl Stop & Glo reagent was added and the reading was taken which corresponds to renilla activity. The process was repeated for each sample in a fresh plate each time. The luciferase activity of each well was normalized by the pRL-SV40 Renilla value using the formula: Ln = L/R (Ln: normalised luciferase activity; L: luciferase activity reading and R: Renilla activity reading). Transcriptional activity of the control, pGL3-Basic, was used to further standardize Ln using the formula: RLU = Ln/pGL3-basic (RLU: relative luciferase unit) (Promega UK Ltd., Southampton, UK).

2.2.7 Stable transfection

About 0.5-1 million cells/well were cultured in 6 well-plates with 2 ml medium overnight to about 70-80% confluence. Transfection solution was

prepared with 10 μg of DNA and 10 μl lipofectamine 2000 by mixing together for 20 minutes, the cells were sub-cultured at 1:10 ratio in fresh medium with adding packing DNA for 48 hours transfection. DMEM medium with 1000 μg/ml G418 or/and 150 μg/ml hygromycin was used as a selection agent. The cells were allowed 7-10 days culture for the growth of cell colonies and were routinely checked every two days. When the colonies were formed, the selective colonies were picked up by trypsinised and the single colony was transferred to T25 cm^2 flask containing a selective medium to enlarge the population and used for further test.

2.2.8 Confocal microscopy, image analysis

Cell culture

Cells were harvested by trypsinisation.$5x10^4$ cells were plated on a sterile 8-well chamber slide over night at 37℃ for immumofluorencence experiment. Dose-dependent drug treatment or time-dependent treatment was conducted on the following day.

Fluorescence labelling of cells

The cells were washed twice with PBS gently to avoid the cells detached from the surface of the chamber slide, and then the cells were fixed by adding 500 μl of methanol/acetone (Volume Ratio 1:1) for 5 minutes. The fixed cells were washed twice with PBS, then the cells were blocked with 5%

BSA for 30 minutes at room temperature to block non-specific binding of immunoglobulin. The cells were incubated with the primary antibodies for 1 hour at room temperature, washed three times with PBS and then labelled with the secondary antibodies for 1 hour in the dark, then the cells were washed three times in PBS. Coverslips were mounted onto slides using anti-fade mounting medium. Images were captured using laser-scanning microscope (Carl Zeiss Laser Scanning Systems LSM 510).

2.2.9 RNA extraction

1 x 10^6 cells were harvested and washed once with cold 1 x PBS, cell pellets were lysised in trizol in 1.5 ml eppendorf for 5 minutes at room temperature. The samples were vigorously shaked shortly with 250 µl of chloroform and then the samples were incubated for 5 minutes at room temperature. After centrifuge the samples at 10,000 g for 5 minutes, the aqueous phase was carefully pipetted off to another 1.5 ml eppendorf. The aqueous phase and 550 µl of isopropanol were mixed gently for 5 minutes at room temperature, and then mixtures were centrifuged again at maximal speed (14,000g) for 20 minutes. The isopropanol was discarded from mixtures and 1 ml of 75% EtOH in DEPC treated H_2O was added for washing the pellet barely visible at the base of tube. The pellets were re-centrifuged at 8,000 g for 5 minutes and air-dried. Finally, the RNA pellets were dissolved by adding 30-50 µl (depending on yield) of either DEPC

treated TE buffer or water. RNA concentration was determined spectrophotometrically by measuring absorbance at 260 nm (1 absorbance = 40 ng/ml RNA). The 260/280 ratio should be greater than 1.8. If less than 1.5-1.6 or so, the RNA is partially degraded. Lower ratios also suggest DNA or thiocyanate contamination. The concentration is essentially the equivalent of the OD at 260 nm (in μg/μl).

2.2.10 Reverse transcriptase polymerase chain reaction

In this study, a promega access RT-PCR system kit was used to perform the experiment. This system is one step RT-PCR in a single tube in which a reverse transcriptase produces first a strand of cDNA from RNA, then a thermostable DNA polymerase produces a second strand of DNA and amplifies the specific DNA of interest. A ratio of 1:2 oligodT primers: RNA template was used in a volume of 25 μl containing 5 μl of 5 x AMV/Tfl reaction buffer, 1 μl of TAMV-reverse transcriptase, 1 μl of Tfl DNA polymerase, 2 μl of 25 mM MgSO4, 1 μl of dNTPs (10 mM of ATP, CTP, GTP and GTP) and nuclease free water up to 25 μl. Reverse transcripton was performed at 45℃ for 45 minutes, following inactivation of AMV RT and denaturation of the RNA/cDNA hybrid by incubation at 94℃ for 2 minutes. The human housekeeping gene GAPDH was used as the RNA loading control, which was amplified by the same RT-PCR

system. The table 2.2 shows the primers, which I used in RT-PCR in my study.

Amplification was performed using standard cycling parameters: 94℃ 30 seconds for denaturation, 58℃ 1 minute for annealing and 68℃ 1 minute for extension. After the final PCR cycle the final extension was at 68℃ for 5 minutes and then kept at 4℃ until further analysis. The RT-PCR products were separated on a 1% agarose gel and the bands visualized and photographed under UV light.

Table 2.2 Primers

Target Primer	Product Size	Forward primer 5' - 3'	Reverse primers 5' - 3'
GAPDH	226bp	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
ALDH1A1	154bp	TGTTAGCTGATGCCGACTTG	TTCTTAGCCCGCTCAACACT
ALDH1A3	150bp	TCTCGACAAAGCCCTGAAGT	TATTCGGCCAAAGCGTATTC
ALDH2	193bp	AACCAGCAGCCCGAGGTCTT	AAGGTGAGCCCAGCTGGAAG
ALDH3A1	229bp	TGTTCTCCAGCAACGACAAG	CTGACCTTCAGGCCTTCATC

2.2.11 Purification of PCR products

PCR products were purified using a StrataPrep® PCR Purification kit. An equal volume of DNA-binding solution was added to the aqueous portion of the PCR products. Transfer PCR product-DNA-binding-solution mixture to a micro-spin cup and spin at 12,000 g for 30 seconds. The PCR products remaining in the fiber matrix of the micro-spin were washed with 1× washing buffer then eluted using 50 µl of elution buffer.

2.3 Molecular biology protocols

Preparation of LB plates

LB medium was prepared by dissolving 25 g LB broth powder and 20 g agar into 1 L of deioned water. This was autoclaved and allowed to cool to 50 ℃ prior to the addition of ampicillin (50 μg/ml) or kanamycin (50 μg/ml). The medium was mixed to ensure equal distribution of antibiotic and approximately 15 ml poured per 10 cm^2 Petri dish. The plates were left to set at room temperature before being stored at 4 ℃.

Transformation of competent bacterial cells

Component bacteria DH5α was purchased from invitrogen and kept at -80°C. An aliquot of 50μl competent bacteria DH5α was allowed to thaw on ice. 10 ng of DNA was added and incubated on ice for 15 minutes. The cells were subjected to a heat shock at for 42 ℃ 90 seconds and returned to ice for 2 minutes. Added 1 ml of SOC medium to the cells and incubated at 37 ℃ for 1 hour in a shaking incubator. One hundred microliters of the reaction was spread onto a LB plate containing the appropriate antibiotic and incubated overnight at 37 ℃.

Preparation of plasmid DNA

Mini-preps

Mini-prep purification was carried out using Qifilter miniprep kit following the manufactures instructions. Cells were harvested by centrifugation at 12,000 g for 5 minutes and re-suspended by pipetting in 100µl of re-suspension buffer I (50 mM Tris-HCl, 10 mM EDTA, pH 8.0 containing 100 µg/ml RNase A). Cells were treated with 100µl of lysis buffer II (200 mM NaOH, 1% (w/v) SDS), mixed and incubated for 5 minutes with gently up and down. Lysis was terminated by the addition of 100µl of neutralisation buffer III (3 mM potassium acetate, pH 5.5). After centrifuging for 10 minutes at 12,000 g, the supernatants of cell lysates were transferred to mini-prep columns. The columns were centrifuged for 1 minute at 12,000 g and washed twice with 750 µl wash buffer (1.0 mM NaCl, 50 mM Tris-HCl, pH 7.0, 15% (v/v) isopropanol). After washing, bound DNA was eluted into a clean 1.5 ml eppendorf tube with 100 µl DNase, RNase-free H_2O.

Maxi-preps

The Qiagen Qiafilter kit was used to produce larger scale DNA samples. Purification of DNA was carried out according to the manufactures instructions. A 350 ml culture of transformed bacteria was pelleted by centrifugation for 15 minutes at 6,000 g at 4℃. The cell pellet was re-suspended in 10 ml of pre-cold buffer P1 (50 mM Tris-HCL pH 8.0, 10mM EDTA, 100 µg/µl Rnase A). 10 ml buffer P2 (200 mM NaOH, 1% (w/v)

SDS) was added to the cells with gently up and down for incubating 10 minutes at room temperature. Neutralized the reaction by adding 10 ml of buffer P3 (3.0 M potassium acetate pH 5.5) and the solution was immediately applied to a QIAfilter cartridge and left for 10 minutes at room temperature to settle. Meanwhile, a Qiagen tip 500 was equilibrated by the addition of buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol). After 10 minutes, the lysed cells were added onto the equilibrated tip and allowed to filter through. The column was washed with 60 ml of buffer QC (1.0 M NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol). The DNA was eluted by the addition of 15 ml of buffer QF (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% (v/v) isopropanol) to the tip. After adding 10.5 ml isopropanol, the DNA was precipitated by centrifuging at 12,000 g for 30 minutes at 4℃. The DNA pellet was washed with 5 ml of room temperature 70% (volume ratio) ethanol and then centrifuged for 15 minutes at 12,000 g at 4℃. The supernatant was carefully removed and the pellet allowed to air dry prior to re-suspension in 0.5-1 ml of DNase, RNase-free H_2O.

Quantification of DNA

DNA measurement was performed by examining the absorbance of a 1:40 dilution of the sample at 260 nm. An A260 value of 1 unit was taken to

correspond to 50 μg/ml of double stranded DNA. The sample concentration could be determined using the following formula.

Sample concentration = OD 260 x dilution factor (40) x 50 μg/ml

Digestion of DNA with restriction endonucleases

One unit of restriction endonuclease activity was capable of completely digesting 1 μg of DNA depending on the manufacturer. 1 μg of DNA digests were prepared using the appropriate restriction enzyme (1-2 units) with a specified buffer providing by the manufacturer provided in a final volume of 20 μl. Reactions were incubated at 37℃ for a minimum of 2 hours.

Agarose gel electrophoresis of DNA

Digested DNA samples or PCR reactions were examined using gel electrophoresis. Samples were diluted in 5 × DNA loading buffer. 1.0% (w/v) agarose gel was prepared by mixing agarose with 1 × TAE and 2.5 mg/ml ethidium bromide. The gels were set in a horizontal gel tank and once set immersed in 1 × TAE. Samples were loaded and the gels run at 100V for 1 hour. The DNA fragments were visualized under ultraviolet light. The size of each fragment was assessed by comparison with DNA molecular markers.

DNA Purification from agarose gels

The required DNA fragments were excised from the gel with a sterile disposable scalpel blade inside the UV transilluminator hood. Excised gel fragments were transferred to a sterile tube. The fragments were purified using the QiaQuick gel extraction according to the manufacturer's instructions. Excised DNA fragments were dissolved in buffer QG followed by the addition of isopropanol at 37℃. The solution was loaded onto a purification column. After centrifugation, DNA was eluted from the purification column using 30 μl of sterile water.

Ligation of DNA

When plasmid DNA and/or PCR fragments had been digested and purified as described above, ligation of digested PCR or DNA fragments to the digested vector DNA was achieved using a Rapid DNA ligation kit. A molar ratio of 1:3 to 1:8 vector, PCR product/DNA fragment was used in a volume of 10 μl containing 1 unit of ligase and the supplied buffer. The reaction was incubated at room temperature for 1 hour or at 4℃ overnight.

DNA sequencing

DNA samples were posted to the Sequencing Service (School of Life Sciences, University of Dundee, Scotland) for sequencing analysis.

2.4 Statistical Tests

All statistics used in analysing the data were conducted using Microsoft Excel 2010 data analysis tools. The cut-off for statistical significance between samples was set to $p=0.05$. Any statistical tests with $p<0.05$ defines 'the results between the samples are statistically different', $p<0.001$ 'the results between the samples are statistically very different', and $p\geq0.05$ defines'there are no statistical significance between the samples'. The statistical test's-test: paired two samples for means' was used to determine the statistical significance between BC cell lines that were tested under the exact same conditions. The statistical test "t-test": Two-sample assuming unequal variance' was used to determine the statistical significance of the same BC cell line that was tested under different conditions. The statistical test "Anova: single factor" was used to determine the statistical significance of the same BC cell line that was tested under different conditions.

3. Disulfiram targeted breast cancer stem cells via Reactive Oxygen Species-MAPK and NFκB pathways

3.1 Introduction

BC is the most common invasive cancer in women globally and the second most common female cancer in the UK. Although the median survival of BC has been significantly improved in the last three decades, the survival rate of the advanced BC remains very poor with only a median 2-3 years of survival for the patients after combinational treatment (Statistical Information Team, CRUK, 2010). Development of radio- and chemo-resistance is one of the major obstacles to the success of BC therapeutics. Chemotherapeutic agents induce DNA damage and trigger cancer cell apoptosis. Meanwhile, these genetic insults also activate the anti-apoptotic pathways in cancer cells, which protect cancer cells from drug-induced apoptosis.

NFκB is one of the major chemoresistance-related anti-apoptotic factors. For the past 25 years, NFκB has served as a model for inducible transcription factors, and plays the most important role in the development

of various kinds of cancer. In comparison with their normal counterparts, many human cancer cells including BC possess higher levels of the constitutive NFκB activity which can be further induced by some anticancer drugs e.g. PAC, dFdC and Dox. Constantly activated NFκB triggers a series of molecular reactions including up-regulation of anti-apoptotic protein-encoding genes (Dutta *et al*., 2006) leading to cancer chemoresistance. In drug-resistant cancer cell lines, high levels of NFκB activity have been identified (Dutta *et al*., 2006). Ectopic overexpression of NFκB can block anticancer drug-induced apoptosis (Wang *et al*., 1999; Wang *et al*., 1998; Wang *et al*., 2004). Previous studies in our lab demonstrate that 5-FU and dFdC-resistant cancer cell lines possess higher NFκB activity (Wang *et al*., 2003). Overexpression of p50 and p65, the two subunits of NFκB, results in increased NFκB activity and induces 5-FU and dFdC resistance (Guo *et al*., 2009; Wang *et al*., 2004). Although NFκB is an attractive molecular target and plays a critical role in therapeutic intervention, inhibition of NFκB alone can only induce limited cell death instead of eliminating all the cancer cells. Disappointing clinical trial outcomes from using a NFκB inhibitor in the treatment of metastatic BC patients (Cresta *et al*., 2008; Yang *et al*., 2006) indicate that BC chemotherapy cannot be efficiently improved by only targeting NFκB pathway. This phenomenon indicates that BC chemoresistance is a multifactorial molecular event in which many biological factors and

signalling pathways are involved. Therefore targeting a single pathway will not be able to reverse chemoresistance and improve therapeutic outcomes in clinic.

ROS are a group of oxygen-containing chemical species normally generated from mitochondrial respiratory chain reaction with reactive chemical properties (Gupte *et al*., 2009). Many chemotherapeutic agents can induce high levels of ROS activity, which damages DNA, protein and lipid membrane leading to apoptosis. In comparison with normal tissues, cancer cells generally possess high ROS activity (Fruehauf *et al*., 2007). It has been suggested that anticancer drugs can further induce ROS generation and exhaust the cellular antioxidant capacity, pushing cancer cells over the tolerated ROS threshold and leading to apoptosis (Lopez-Lazaro *et al*., 2007).

Mitogen-activated protein kinase (MAPK) pathways regulate a vast array of physiological processes, such as growth, proliferation, differentiation, migration and apoptosis. MAPK enzymes are regulated by a characteristic phosphorelay system in which a series of three protein kinases phosphorylate and activate one another. To date, six different groups of MAPKs have been characterized in mammals, extracellular signal-regulated kinase (ERK) 1/2, ERK3/4, ERK5, ERK7/8, c-Jun N-terminal kinase (JNK) 1/2/3 and the p38 isoforms α/β/γ(ERK6)/δ (Schaeffer *et al*.,

1999). Here, I will discuss three well-characterized subfamilies of MAPK (ERK, JNK and p38) pathways which will be investigated in my study.

The ERK pathway is the most studied mammalian MAPK pathway which is deregulated in one third of all human cancers and linked to many other aspects of the tumour phenotype (Junttila *et al*., 2008). ERK signalling is activated by numerous extracellular signals including growth factors and mitogens, which are particular relevance to cancer. The ERK pathway is initiated through the activation of the small G-protein Ras, which is a membrane-bound protein activated through the exchange of bound GDP to GTP. Phosphorylation of Ras recruits cytoplasmic Raf (Raf-1, B-Raf and A-Raf) to the cell membrane for activation. The activated Raf will regulate phosphorylation of MEK (MEK1 and MEK2) followed by the activation of ERK (ERK1 and ERK2). Activated ERK1 and ERK2 can translocate to the nucleus, and then they activate several transcription factors, such as c-Fos, ATF-2, Elk-1, c-Jun, and c-Myc. The function of the ERK pathway has long been associated with cell growth, cell proliferation, and survival.

Sustained ERK signalling promotes phosphorylation and stabilization of cyclin D1, c-Fos, c-Jun and c-Myc genes to push cell-cycle entry, and repress the expression of genes which inhibit proliferation (Yamamoto *et al*., 2006). Furthermore, high expression of ERK can lead to cell-cycle arrest by inducing the expression of CDK-inhibitor proteins such as p21

and p27 (Sewing *et al*., 1997; Woods *et al*., 1997; Mirza *et al*., 2004). ERK1 and ERK2 mediated survival signalling has been demonstrated mainly through activation of RSK kinase. Phosphorylation of RSK inactivates the pro-apoptotic protein BAD. Meanwhile, activates the transcription factor CREB, which promotes cell survival through transcriptional up-regulation of anti-apoptotic Bcl-2, Bcl-xL, and Bcl-1 proteins (Ballif *et al*., 2001; Bonni *et al*., 1999). In addition, ERK1 and ERK2 activity can suppress Fas-mediated apoptosis by inhibiting the formation of the death-inducing signalling complex (Holmstrom *et al*., 2000).

The JNK pathway is mainly activated by cytokines or cellular stress. These stimuli activate JNKs through several upstream kinases (MAPKKKs) phosphorylated, such as DLK, ASK1, MLK-3, MKKK1-4, TAK-1, and TPL-2 (Davis *et al*., 2000; Weston *et al*., 2007). In order to fully activate JNK, activation of both MKK4 and MKK7 are needed at MAPKKs level. Thereby JNK is activated through phosphorylation of MKK4 and MKK7. JNK then activates downstream transcription factors including c-Jun, ATF-2, Elk-1, MEF-2c, p53, and c-Myc in the nucleus. Also, JNK has other non-transcriptional substrates such as Bcl-2 and Bcl-xL (Yamamoto *et al*., 1999). The JNK family is encoded by JNK1, JNK2 and JNK3. Like all other MAPKs, JNKs are activated through phosphorylation of a tyrosine

and a threonine residue, and share 85% sequence identity and are expressed ubiquitously. Regulation of the JNK pathway is extremely complex; the activity of JNK pathway can mediate apoptosis, proliferation, or survival, depending on the stimuli and cellular conditions. Double knockout JNK1 and JNK2 mice were embryonic lethal due to altered apoptosis during brain development (Sabapathy *et al*., 2001; Tournier *et al*., 2000). JNK3-null mice demonstrated differences in neuronal apoptosis as compared to wild-type mice (Yang *et al*., 1997). The transcriptional factor c-Jun is a component of the AP-1 transcription complex, which is an important regulator of gene expression. Phosphorylation of c-Jun, which is directly activated by JNK, has been demonstrated to play a critical role in Ras-induced tumourigenesis through transcriptional repression of the p53 gene (Smeal *et al*., 1991; Kennedy *et al*., 2003). The inhibition of JNK enhances chemotherapy-induced inhibition of tumour cell growth because of its ability to interfere with DNA repair in response to genotoxic drug, suggesting that JNK may provide a molecular target for the treatment of cancer. The pharmaceutical industry is bringing JNK inhibitors into clinical trials for cancer treatment.

The p38 pathway is strongly activated by inflammatory cytokines and environmental stresses, such as TNFα, IL-1, hypoxia, ultraviolet light, and osmotic stress. The diverse range of signals, such as TAK1, ASK1/2, DLK,

MEKK4, TAO1/2/3 and MLK2/3 can activate the p38 pathway through several MAPKKKs. Phosphorylation of MAPKKKs then activates MEK3 and MEK6, which directly trigger p38 protein. Once active, p38 protein can translocate from the cytosol to the nucleus to activate an array of transcription factors, including ATF-2, CHOP-1, MEF-2, p53 and Elk-1. The p38 protein is represented by four isoforms: p38α, p38β, p38γ, and p38δ. They are characterized by the presence of the conserved Thr-Gly-Tyr (TGY) phosphorylation motif in their activation loop (Kumar *et al*., 2003). The p38 pathway plays a role in the regulation of apoptosis, cell cycle, and differentiation. Stress-induced p38 activation was shown to cause G2/M cell cycle arrest and to regulate the cell cycle through modulation of p53 tumour suppressor proteins (Bulavin *et al*., 2001). p38 is involved in both the activation of p53 and in p53-induced apoptosis and acts as a negative regulator of cell cycle progression (Kummer *et al*., 1997; She *et al*., 2001). Furthermore, p38 activity has been reduced in hepatocellular carcinomas in comparison to adjacent normal tissue, with tumour size inversely related to p38 activity (Iyoda *et al*., 2003). In contrast, a few reports showed p38 pathway activity can promote cancer cell growth and survival. For example, a high level of p38 has been observed in some cancer types, such as BC and brain tumour. p38 activity is also involved in the invasiveness of several cancer cell lines and inhibition of p38 activity reduced their proliferation, survival, and invasion (Junttila *et al*., 2007; Johansson *et al*.,

2000). In addition, many chemotherapeutic agents induced apoptosis by p38 activity. Inhibition of p38 activity can enhance apoptosis in response to DNA-damaging agents such as Dox and CDDP in as well as microtubule-disrupting agents such as taxol, vincristine and vinblastine (Deacon *et al*., 2003; Losa *et al*., 2003; Lee *et al*., 2006).

The most important function of MAPKs is to control cellular responses to the environment and regulate gene expression, cell growth, and apoptosis, which is related to many human diseases. The ERK, JNK, and p38 pathways are all molecular targets for drug development, and inhibitors of MAPKs will undoubtedly be one of the next group of drugs developed for the treatment of human disease.

Many conventional anticancer drugs induce ROS generation and trigger cancer cell apoptosis via ROS-MAPK pathway. However anticancer drug-induced ROS activation also triggers the expression and activation of a number of anti-apoptotic factors including NFκB that dampen the ROS-induced cytotoxic effect (Nakano *et al*., 2006). Interestingly, sustained JNK activity is necessary for cellular homeostasis, whereas strong stress ROS-induced apoptosis is highly reliant on the persistent activation of pro-apoptotic MAPK pathways (JNK and p38) (Nakano *et al*., 2006). JNK and NFκB signalling often play opposing roles in cancer, activation of NFκB signalling can lead to the suppression of apoptosis in contrast to JNK

signalling. Prolonged JNK activation was found to promote apoptosis, suggesting that genes are induced by TNFα in an NFκB-dependent fashion normally block JNK activation. In response to TNFα, the anti-apoptotic effect of NFκB has been shown to be mediated by the induction of genes that can repress JNK activity (Lee *et al*., 2006). ROS have emerged as bridging molecules mediating the crosstalk between NFκB and JNK. A number of data demonstrated that ROS directly activate various kinases, including ASK1, MEKK1, and EGFR, which in turn activate the MAPK cascades. NFκB down-regulates JNK activation by suppressing TNFα induced ROS accumulation (Kumar *et al*., 2003). TNFα induces JNK activation in wild-type cells, whereas TNFα induces ROS accumulation leading to prolonged JNK activation in NFκB activation-deficient cells. Prolonged JNK activation is inhibited by pre-treatment of cells with antioxidants such as N-acetyl cysteine (NAC), suggesting that the mechanisms of early/transient and prolonged JNK activation are qualitatively different (Kumar *et al*., 2003). ROS are toxic in cells at certain levels, the correct cellular response to ROS production is consequently critical in order to prevent further oxidative damage, and to maintain cell survival. However, when too much cellular damage has occurred, ROS can trigger both apoptotic and necrotic cell death through the JNK and p38-MAPK pathways. The JNK pathway is one of the main signalling pathways that intersect with NFκB with regard to ROS and cell death. In most cases,

the expression of NFκB target genes typically promotes cellular survival (Gloire *et al*., 2006). Therefore ROS modulate an NFκB response. Meanwhile, NFκB target genes would attenuate ROS to promote survival. Both JNK and p38 pathways are response for pro-apoptotic which regulate downstream genes, as a feedback effect, some anti-apoptotic factors such like Bcl-2 which is NFκB downstream target genes will be triggered to prevent cell death. Because of the cross-talk between NFκB and ROS-MAPK pathways, singly targeting either pathway may not be sufficient for inducing cancer cell apoptosis. Therefore, identification of small molecules which simultaneously activate the ROS-MAPK pro-apoptotic pathway and inhibit ROS-induced anti-apoptotic pathways may improve BC chemotherapy.

DS is a commercially available anti-alcoholism drug (Johansson *et al*., 1992) used in clinic for more than 60 years. Our group has previously demonstrated that DS inhibits NFκB activity and enhances 5-FU- and dFdC-induced apoptosis in drug-sensitive and resistant colon cancer cell lines (Guo *et al*., 2009; Wang *et al*., 2003). DS also sensitises the cytotoxicity of cyclophosphamide, CDDP and radiation *in vitro* and protects normal cells in kidney, gut and bone marrow *in vivo* whilst increasing the therapeutic index of a wide range of cytotoxic drugs (Bodenner *et al*., 1986; Evans *et al*., 1982; Hacker *et al*., 1982). The

molecular anticancer mechanisms of DS are still not very clear. Previous publications indicate that DS targets the cancer cells in a Cu dependent manner (Cen *et al*., 2004; Cen *et al*., 2002; Chen *et al*., 2006; Nobel *et al*., 1995).

Cu is an essential trace element for humans and only high intracellular concentrations of Cu have been found to generate ROS in cells which can damage DNA, protein and lipid membranes eventually resulting in apoptosis (Gupte *et al*., 2009). Cu-induced cell death is associated with an increase in ROS generation (O_2^-) through reduction of Cu^{2+} to Cu^{1+} in GSH to GSSH (Morrison *et al*., 2010). This would eventually deplete the glutathione pool leading to accumulation of high ROS levels and cell death. Cu intake is tightly controlled by Ctr1 as an essential function. This is the main reason why Cu alone does not show cytotoxicity in the cancer cells. DS/Cu is a strong ROS inducer (Nobel *et al*., 1995) and proteasome-NFκB pathway inhibitor (Chen *et al*., 2006). A combination of DS with Cu is supposed to target cancer cells by simultaneously tackling both ROS and NFκB.

Cancer cells contain a very small fraction (1%) of CSCs which are relatively quiescent and express multidrug resistant and anti-apoptotic proteins (Dalerba *et al*., 2007; Marques *et al*., 2010; Storci *et al*., 2010). The importance of CSCs in chemoresistance has been attracting more and

more attention from cancer research and clinics. Conventional anticancer drugs target the proliferating and differentiated tumour bulk but fail to eradicate the CSCs, which become the source of tumour recurrence. Therefore, targeting CSCs becomes one of the new strategies for reversing chemoresistance and drug development. ALDHs are functional markers of normal and breast cancer stem cells (BCSCs) (Ginestier *et al*., 2007; Marcato *et al*., 2011). It was recently reported that targeting the ALDH1A1 gene could kill the ovarian CSCs (Landen *et al*., 2010). DS is a specific inhibitor of ALDHs (Lam *et al*., 1997). Therefore, it may also be an inhibitor of BCSCs.

The aim of the study in this chapter is to determine the anticancer activity of DS supplemented with physiological concentration of Cu. I also examined the chemosensitizing effect of DS/Cu on PAC induced cytotoxicity in a panel of BC cell lines.

3.2 Methods

General methodologies have been described in the chapter II. The following are the specific methods used in this study.

3.2.1 MTT and CI-isobologram

Overnight-cultured T47D, MCF7 and MDA-MB-231 cells were exposed to various concentrations of PAC, DS+$Cu_{1\,\mu M}$ or in combination of PAC and DS+$Cu_{1\,\mu M}$ at a constant PAC: DS ratio of 62.5:1 for 72 hours. The cells were then subjected to MTT analysis as described above. The combinational cytotoxic effect between PAC and DS/$Cu_{1\,\mu M}$ was determined using CI-isobologram analysis (CalcuSyn software, Biosoft, Cambridge, UK). The combination index (CI) was determined by mutually exclusive equations.

3.2.2 Reactive Oxygen Species activity detection

The intracellular ROS levels were determined using 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) probe (Invitrogen, Paisley, UK). T47D, MCF7 and MDA-MB-231 BC cells (1×10^6) were cultured in 24-well plates with 1 ml of serum- and phenol red-free DMEM medium (Sigma) containing 20 μM of H_2DCFDA. Fluorescence was measured in 96-well plates at excitation 490 nm and emission 520 nm using a Fluoroskan Ascent fluorometer (Thermo Scientific, Northumberland, UK).

3.2.3 Clonogenic assay

T47D, MCF7 and MDA-MB-231 BC cells (5×10^4/well in 6-well plates) were exposed to a designated concentration of DS/$Cu_{1\mu M}$, PAC or PAC + DS/$Cu_{1\mu M}$ for 24 hours. The cells were collected and further cultured for 7 (MDA-MB-231 and MCF7) to 14 (T47D) days in 6-well plates containing drug-free medium at a cell density of 2.5×10^3/well. Clonogenic cells were determined as those able to form a colony consisting of at least 50 cells.

3.2.4 Detection of aldehyde dehydrogenases positive population

The ALDH positive population in drug-treated T47D, MCF7 and MDA-MB-231cell lines was detected by ALDEFLUOR kit (StemCell Tech., Durham, NC, USA) following the supplier's instruction. The cells (2.5×10^5) were analyzed after being stained in ALDH substrate containing assay buffer for 30 min at 37℃. The negative control was treated with diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor.

3.2.5 *In vitro* mammosphere culture

The T47D, MCF7 and MDA-MB-231 BC cells were cultured in ultra-low adherence 6-well plates (Corning, MA, USA) containing 2 ml of stem cell culture medium [SCM, serum-free DMEM-F12 supplemented with B27

(Invitrogen, Paisley, UK), 20 ng/ml epidermal growth factor (Sigma), 10 ng/ml basic fibroblasts growth factor (R & D System, Abingdon, UK), and 10 µg/ml insulin (Sigma)] at a density of 10,000 cells/ml. After 7-10 days culture, the mammospheres were photographed and subjected to further treatments.

3.2.6 Flow cytometric analysis of CD24 and CD44 expression

The adherent or mammosphere cells were trypsinised and passed through a 25G needle. The cells (2.5×10^5) were incubated with CD24 and CD44 antibodies (BD Pharmingen, Oxford, UK) for 20 minutes at 4℃. Unbound antibodies were washed off with 2% FCS HBSS (Sigma) and the cells (10,000 events) were examined no longer than 1 hour after staining on a BD Facscalibur.

3.3 RESULTS

3.3.1 The cytotoxicity of disulfiram in breast cancer cells was copper dependent

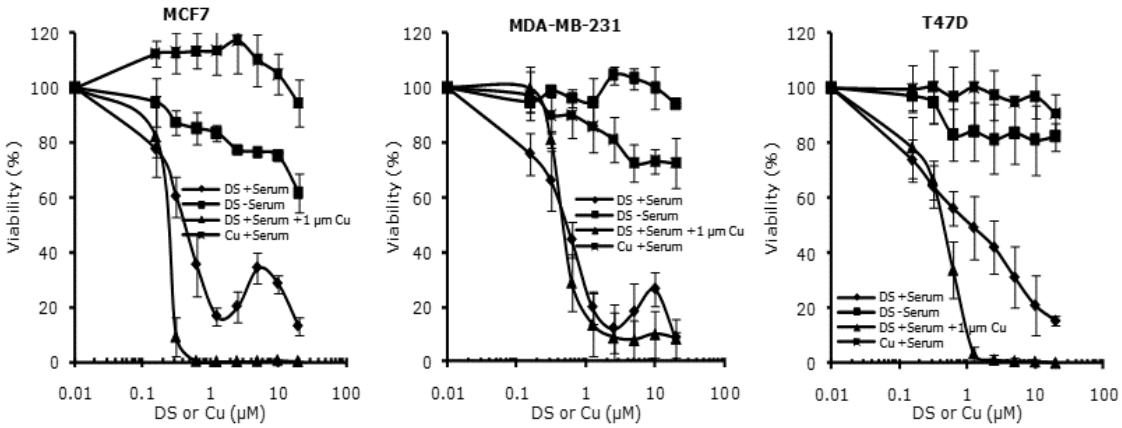
In $CuCl_2$ (1 µM) supplemented medium, DS was highly cytotoxic to BC cell lines (IC_{50}_72h: 110 nM to 476 nM, Fig. 3.1 and Table 3.1). DS was also toxic to cancer cell lines in the complete medium without $CuCl_2$ supplement with higher IC_{50} (456 nM to 1,100 nM, Fig. 3.1 and Table 3.1).

A biphasic effect was observed in 2 out of 3 BC cell lines. The cancer cells appeared to be protected at a higher concentration of DS. DS alone in serum-free medium (to rule out the influence of trace amount of Cu contained in FCS) or Cu alone was not toxic to BC cell lines even at a very high concentration (20 μM). The drug-induced morphological changes are shown in Fig. 3.1. The flow cytometric DNA content analysis demonstrated that cells induced to apoptosis (sub-G1 population) is significant increased after 72 hours of DS/Cu treatment, but similar results could not be found in other groups (Fig. 3.2). The cleaved poly-(ADP-ribose) polymerase (PARP) protein, an indicator of caspase activation, was detected in DS/Cu treated cells. DS/Cu significantly inhibited BCL2 and induced BAX expression respectively therefore increased the BAX/BCL2 ratio in DS/Cu treated cells (Fig. 3.3).

Table 3.1 Cytotoxicity of different treatments to breast cancer cell lines

	MCF7	MDA-MB-231	T47D
IC_{50} (nM)			
DS +Serum	456 (62)	495 (49)	1,100 (87)
DS/Cu	211 (23)	476 (48)	443 (62)
DS –Serum	>20,000	>20,000	>20,000
Cu	>20,000	>20,000	>20,000
PAC alone	4.3 (1.4)	9.3 (0.7)	2.6 (0.3)
PAC + DS/Cu	0.4** (0.1)	0.6** (0.02)	0.7** (0.1)
CI values			
IC_{50}	0.183	0.437	0.446
IC_{75}	0.213	0.436	0.661
IC_{90}	0.265	0.457	0.633

Abbreviations: CI=combination index; Cu=copper; DS=disulfiram; PAC=paclitaxel. The figure represents IC_{50} value from three experiments [mean (SD)]. **Compared with PAC alone, significant difference ($p<0.01$, n=3). The cells were treated for 72 hours. DS/Cu: DS in medium supplemented with 1μM $CuCl_2$; DS-serum: DS in serum-free medium; DS+serum: DS in serum-containing medium. CI: combination index.



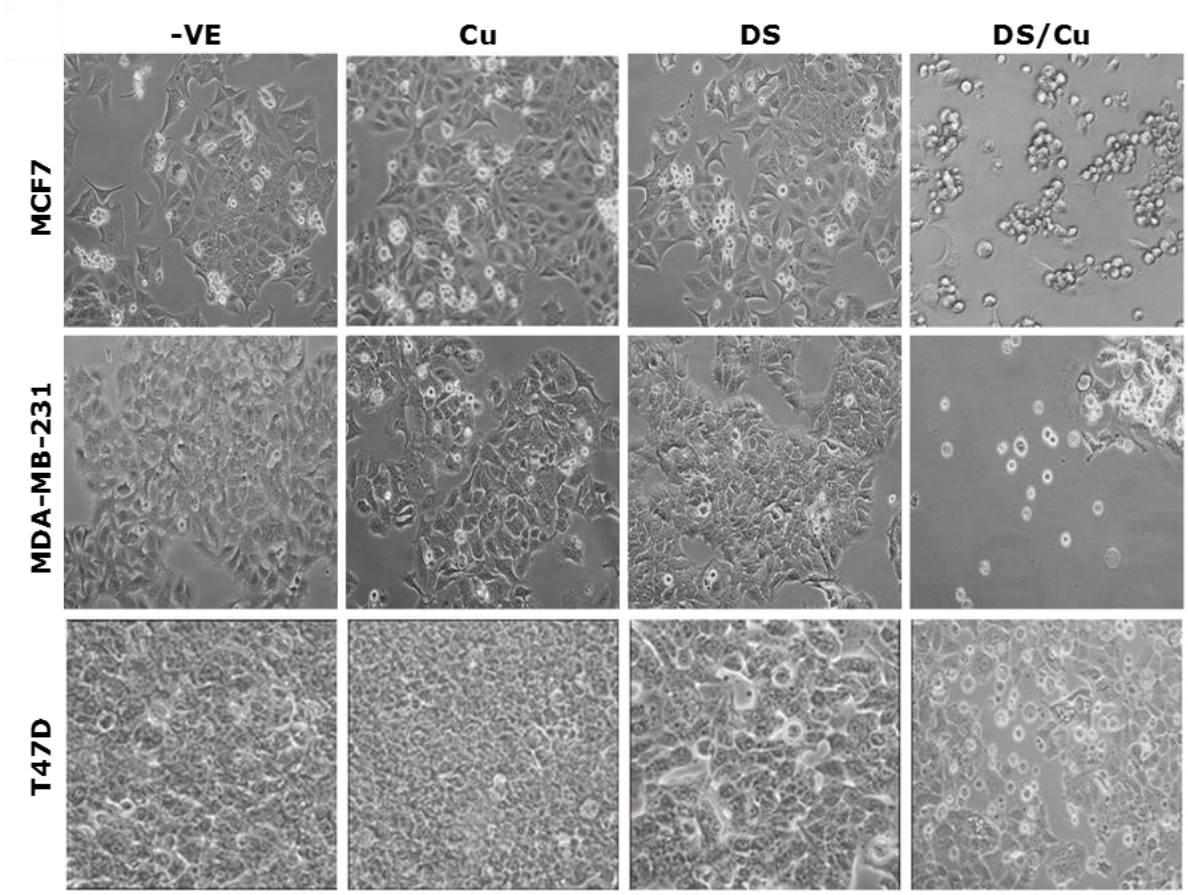


Fig. 3.1 Disulfiram was cytotoxic to breast cancer cells in a copper-dependent manner. MTT cytotoxicity assay. The BC cells were exposed to different treatments for 72 hours. The morphology (× 100 magnification) of BC cell lines after 72 hours drug exposure (DS: 1 μM of DS in serum free medium, Cu: $CuCl_2$ 1 μM, DS/Cu: DS 1 μM + Cu 1 μM).

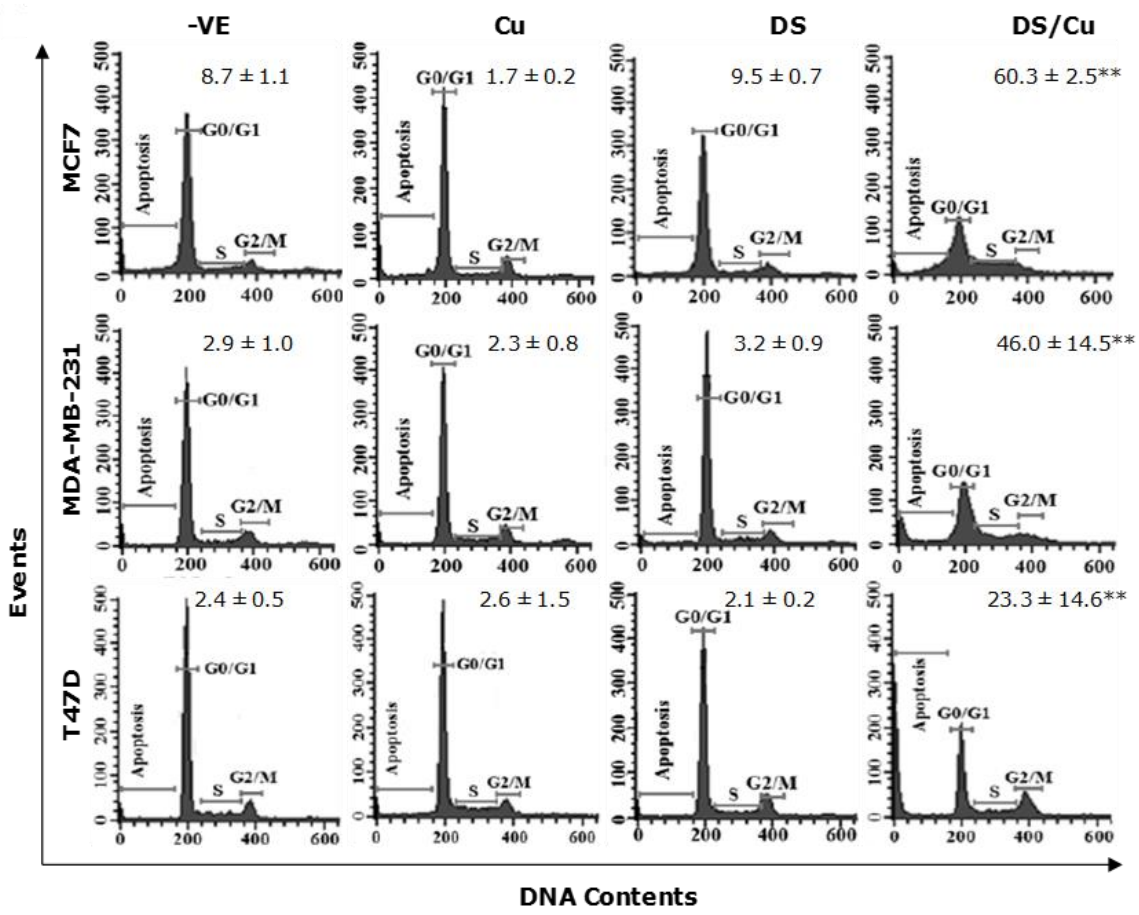


Fig. 3.2 Flow Cytometry Analysis. The DNA contents of BC cells after 72 hours drug exposure (DS: 1 µM of DS in serum-free medium, Cu: $CuCl_{2\ 1\mu M}$, DS/Cu: $DS_{1\mu M}$ + $Cu_{1\mu M}$). The DNA contents in the treated cells (10,000 events) were determined. The sub-G1 population represents the apoptotic cells (** $p<0.01$, n=3).

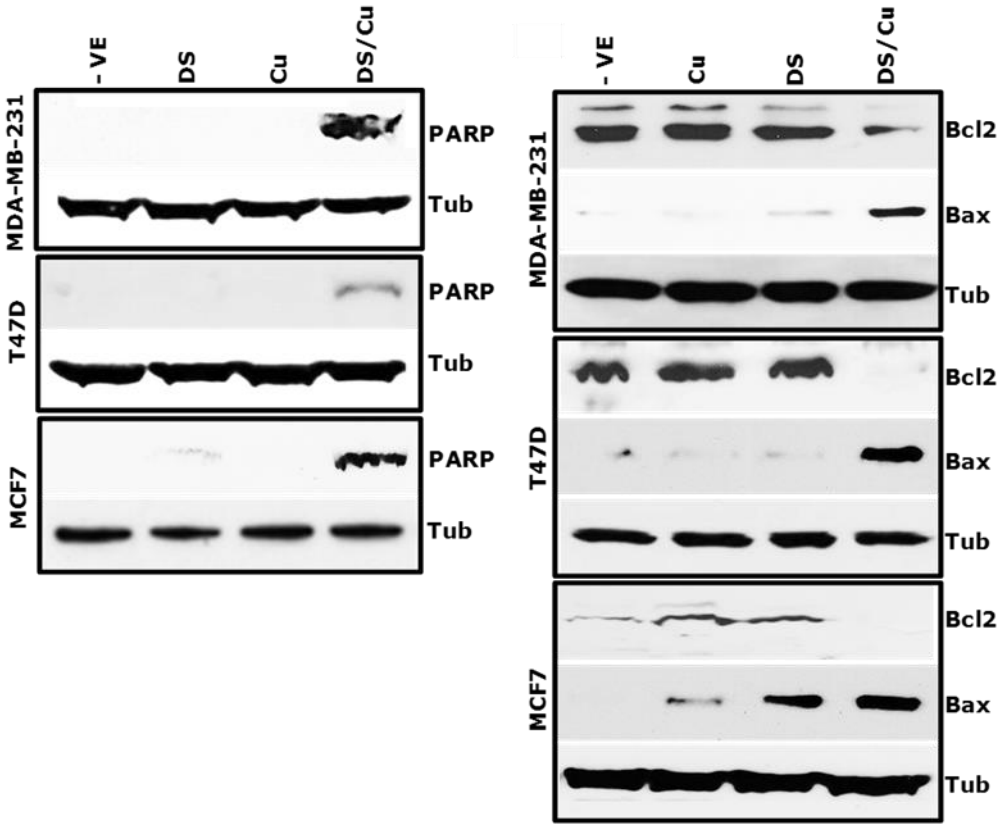


Fig. 3.3 Expression of apoptosis-related proteins. The cleavage of PARP protein and the expression levels of BCL2 and BAX after 72 hours drug exposure were determined by western blot. Tub: α-tubulin used as a loading control.

3.3.2 Disulfiram/copper synergistically enhanced the cytotoxicity of paclitaxel in breast cancer cell lines

In combination with DS/Cu, the cytotoxicity of PAC was significantly enhanced in PAC sensitive (4-16 fold) and resistant (> 1,000 fold) cell lines (Fig. 3.1 and Table 3.1). There was a very strong synergistic effect between DS/Cu and PAC over a wide range of concentrations. In contrast to the slight induction of apoptosis at a low concentration of PAC alone (1 nM),

the proportion of apoptotic cells was massively increased by DS/Cu (DS 100-150 nM/Cu 1 μM) and PAC in combination (Fig. 3.4).

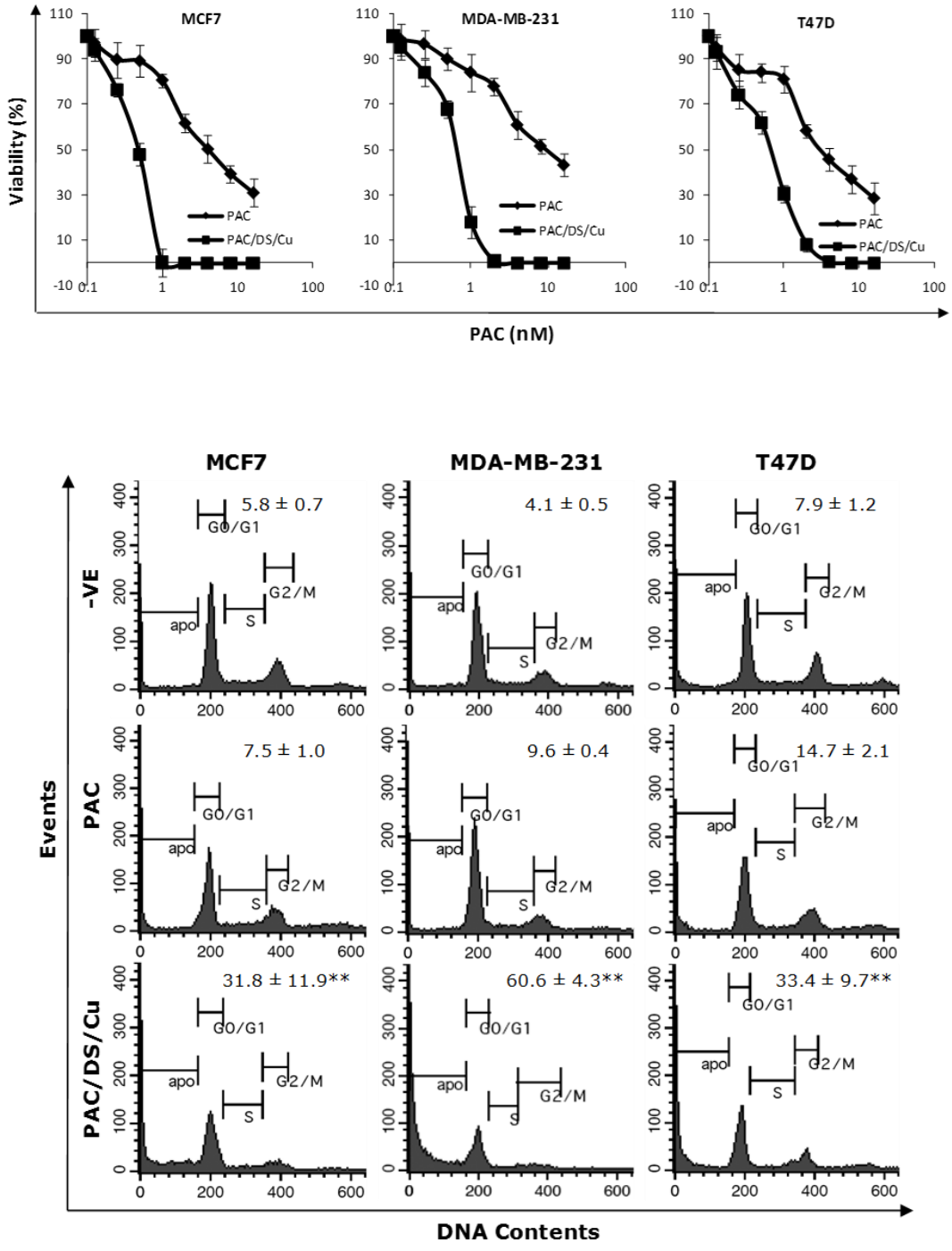


Fig. 3.4 MTT cytotoxicity assay and Flow Cytometry Analysis. Analysis of the combined effect of PAC and DS/$Cu_{1\mu M}$. PAC: DS/$Cu_{1\mu M}$ = 1:62.5. The PAC-induced apoptosis was enhanced by DS/Cu. The DNA contents in the cell lines treated for 72 hours with PAC (1 nM) or PAC plus

DS/Cu (DS: MCF7, 100 nM; MDA-MB-231, and T47D, 150 nM; Cu: $CuCl_2$ 1 μM) were determined by flow cytometry. (** $p<0.01$, n=3).

3.3.3 Reactive Oxygen Species activation was responsible for Disulfiram/copper-induced cytotoxicity

The DS-induced cytotoxicity was highly Cu-dependent. Cu is an active redox metal element, which can trigger ROS generation and induce apoptosis in cancer cells (Morrison *et al*., 2010). ROS may be the mediator for DS/Cu-induced apoptosis. Therefore, I examined the effect of DS/Cu on the generation of ROS in BC cell lines. My study shows that DS significantly induced ROS activity in BC cell lines ($p<0.01$). N-acetyl-L-cysteine (NAC) is a ROS inhibitor, which can block ROS, induced apoptosis (Halasi *et al*., 2013). The DS/Cu induced ROS activity in the BC cell lines was reversed by the addition of a ROS inhibitor, NAC ($p<0.01$; Fig. 3.5). To determine the effect of ROS on DS/Cu-induced cell death, the cytotoxicity assay was performed with or without ROS inhibitor. Fig. 3.5 shows that in line with the change of ROS activity, the DS/Cu-induced cytotoxicity was significantly reversed by the addition of NAC in the culture ($p<0.01$).

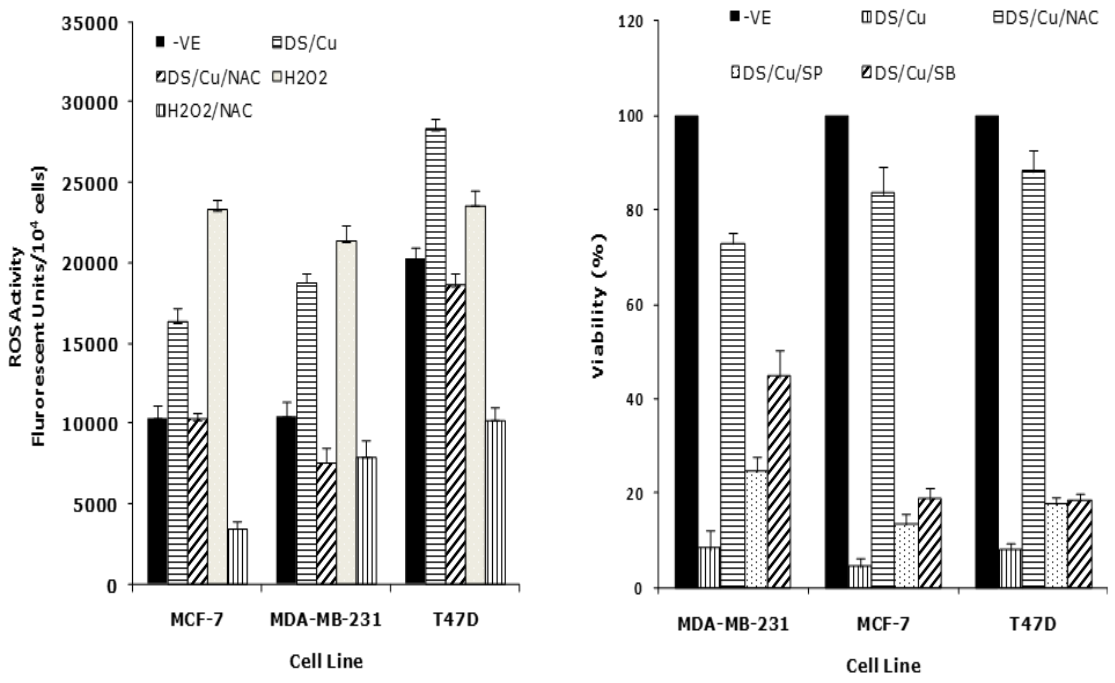


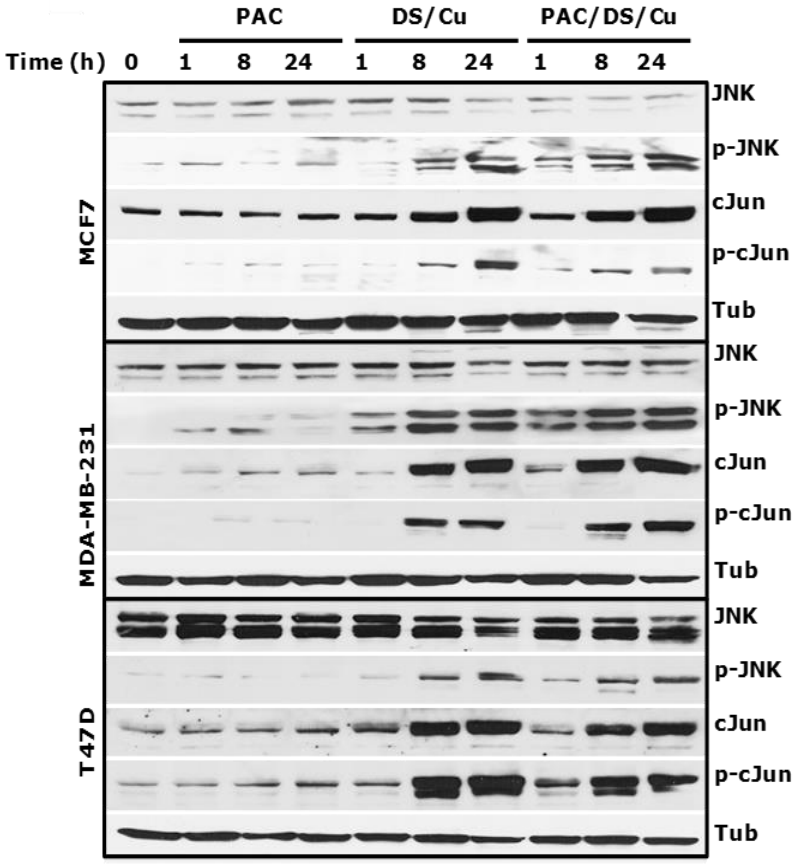
Fig. 3.5 ROS was responsible for disulfiram/copper-induced cytotoxicity. The BC cell lines were loaded with fluorescent dye H_2DCFDA and exposed to $DS_{5\ \mu M}/Cu_{5\ \mu M}$ or DS/Cu plus NAC (10 mM) for 3 hours. The fluorescent strength was detected by fluorometer at Ex 490 nm and Em 520 nm. The effect of ROS and MAPK pathway inhibitors on DS/Cu-induced cytotoxicity. The cancer cells were exposed to $DS_{250nM}/Cu_{1\ \mu M}$, DS/Cu plus NAC (10 mM), JNK inhibitor SP600125 (10 μM) or p38 inhibitor SB203580 (10 μM) for 72 hours and subjected to MTT assay.

3.3.4 Disulfiram/copper triggered persistent activation of JNK and p38 pathways

Fig. 3.6 showed the effect of PAC, DS/Cu and PAC/DS/Cu on the activation of the JNK pathway. Total JNK protein expression was not affected by the above treatments. However, the expression of phosphorylated JNK, c-Jun and total c-Jun was persistently (up to 24 hours)

induced by DS/Cu and PAC/DS/Cu. In contrast, the expression of these proteins was not, or only very mildly, up-regulated by PAC. High levels of phosphorylated p38 were also detected up to 24 hours following DS/Cu and PAC/DS/Cu exposure (Fig. 3.6). To determine the causal relationship between ROS and JNK, p38 pathways, BC cell lines were exposed to DS/Cu for 24 hours with or without the addition of NAC. NAC significantly inhibited or totally blocked DS/Cu-induced c-Jun and p38 phosphorylation (Fig. 3.7).

A



B

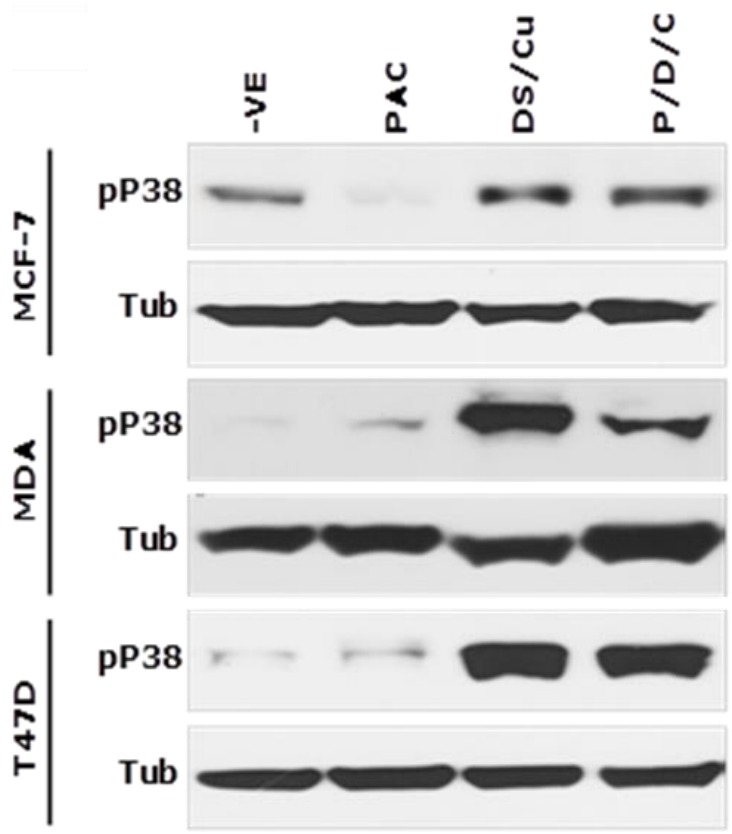


Fig. 3.6 The effect of disulfiram/copper on MAPK pathway. The overnight cultured BC cells were exposed to $PAC_{1\mu M}$, $DS_{1\mu M}/Cu_{1\mu M}$ or $PAC_{1\mu M}/DS_{1\mu M}/Cu_{1\mu M}$ for indicated time lengths. (A) The expression levels and phosphorylation status of proteins in JNK pathways were detected by western blot in different treatment time points (1, 8, 24 hours). (B) Phosphorylation of p38 were detected by western blot after 24 hours treatment.

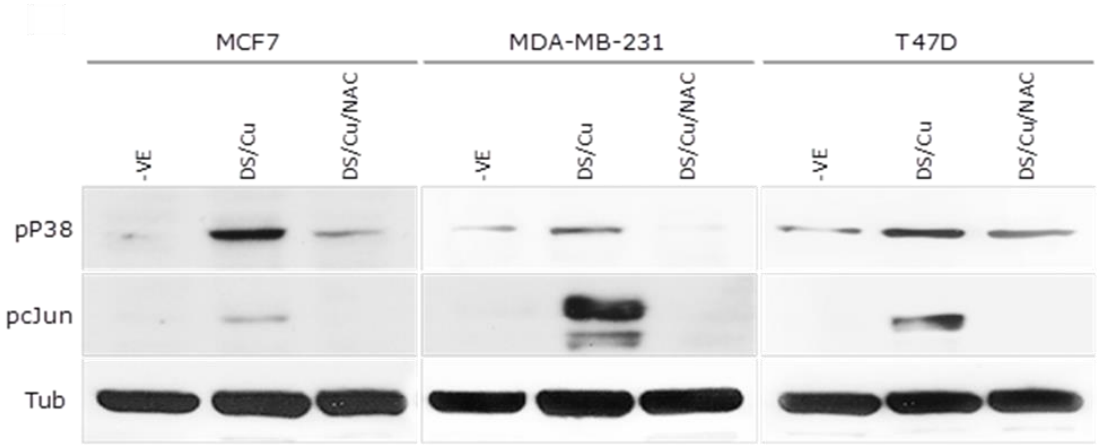


Fig. 3.7 The effect of disulfiram/copper/NAC on MAPK pathway. The activation of JNK and p38 pathways was reversed by NAC. The phosphorylation of c-Jun and p38 in BC cell lines was determined by western blot after exposure to DS/Cu or DS/Cu plus NAC (10 mM) for 24 hours.

3.3.5 Disulfiram/copper inhibited NFκB activity in breast cancer cell lines

NFκB is an ROS-induced transcription factor with strong anti-apoptotic activity which in turn dampens the pro-apoptotic effect of ROS (Nakano *et al*., 2006). Blockage of NFκB activation enhances ROS-induced cytotoxicity. Both PAC and DS/Cu inhibited NFκB DNA binding activity in BC cell lines. The strongest inhibition was observed in the cells treated with PAC/DS/Cu in combination (Fig. 3.8). The inhibition of NFκB transcriptional activity was also detected in PAC, DS/Cu and PAC/DS/Cu treated cells by reporter gene assay (Fig. 3.8).

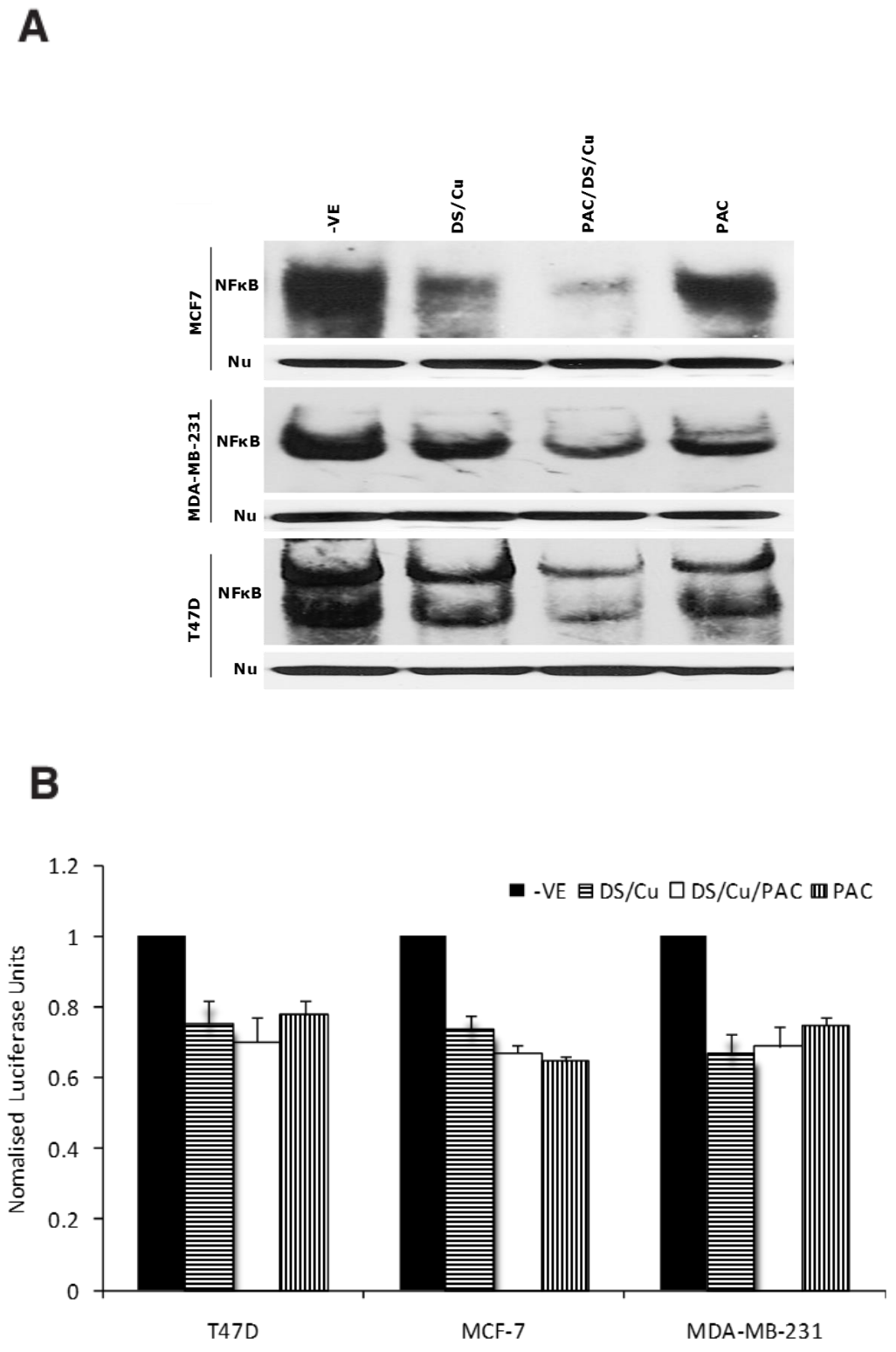


Fig. 3.8 NFκB activity has been inhibited by disulfiram/copper. (A) NFκB DNA binding activity was analysed by EMSA assay. Nu: western blot of nucleolin was used as a protein loading control. (B) The BC cell lines were treated with $PAC_{1\mu M}$, $DS_{1\mu M}/Cu_{1\mu M}$ or PAC/DS/Cu for 24 hours. NFκB

transcriptional activity examined by luciferase reporter gene assay after exposure to $PAC_{1\mu M}$, $DS_{1\mu M}/Cu_{1\mu M}$ or PAC/DS/Cu for 24 hours.

3.3.6 Disulfiram/copper inhibited the clonogenicity in breast cancer cell lines

In this study, I also used clonogenic assays to determine the ability of DS/Cu to induce 'reproductive death' (Franken *et al*., 2006) in BC cells. After 16 hours exposure to PAC (40 nM: 4 to 18-fold higher than IC_{50} concentration), DS (200-250 nM: sub-IC_{50} concentration)/$Cu_{1\mu M}$ or PAC and DS/Cu in combination. The treated cells were collected and cultured in drug free medium for 7-14 days. The colony number was reduced by exposure to PAC, DS or Cu alone. The colony number in PAC, DS and Cu treated groups was decreased which was caused by slow growth of the surviving cells leading to the cell number in some colonies not reaching the counting threshold (50 cells). In contrast, the clonogenicity of BC cell lines was significantly inhibited by DS/Cu and totally eradicated by exposure to PAC plus DS/Cu (Fig. 3.9).

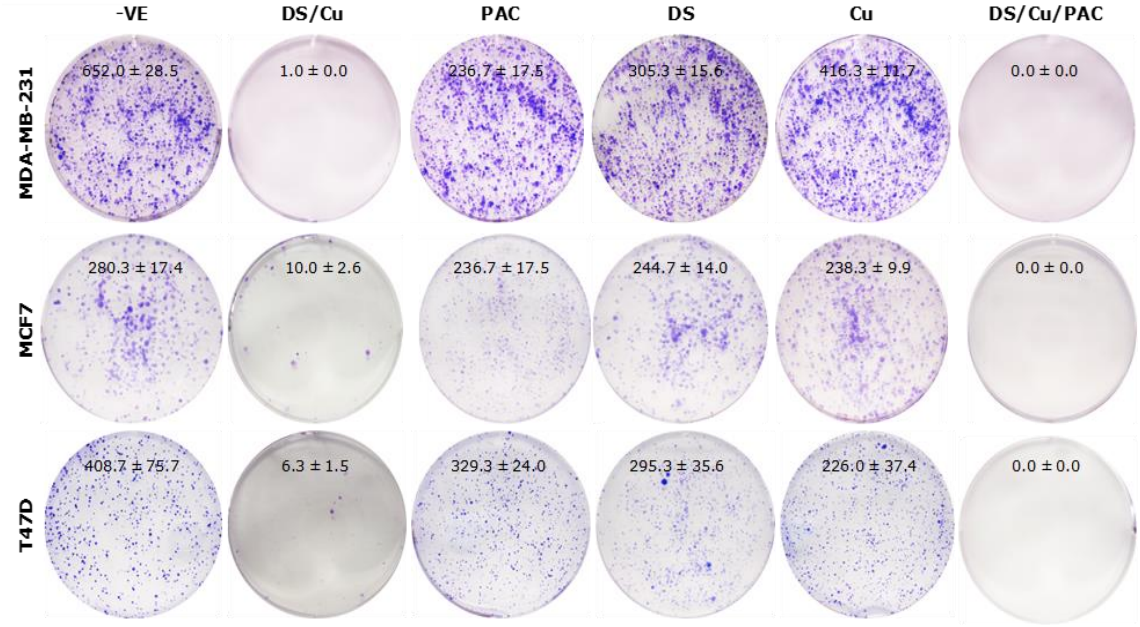


Fig. 3.9 The effect of disulfiram/copper on the clonogenicity. Clonogenic assay. The cells exposed to Cu 1 μM, PAC 40 nM, DS (250 nM for MDA-MB-231 and T47D, 200 nM for MCF7) DS/Cu or PAC/DS/Cu for 24 hours were cultured in drug-free medium in 6-well plates at a cell density of 2.5 × 10^3/well for 7-10 days. The colonies were stained with crystal violet, counted and photographed as described in Materials and Methods.

3.3.7 Disulfiram/copper targeted breast cancer stem cells

Furthermore, we examined the effect of different treatments on CSCs population in MDA-MB-231 and T47D cell lines. The mammosphere formation in both cell lines was completely blocked by exposure to $DS_{1\,\mu M}/Cu_{1\,\mu M}$ or DS/Cu plus PAC_{40nM} for 48 hours but not affected by PAC, DS or Cu alone (Fig. 3.10). To determine the targeting effect of DS/Cu on CSCs, the BCSCs markers in drug-treated mammosphere cells were also analysed. Fig. 3.11 demonstrates that the ALDH positive population in

mammospheres was significantly inhibited by DS/Cu but not affected or even enriched by DS or Cu treatment. In order to determine the effect of DS/Cu on CSCs, the expression status of $CD24^{Low}/CD44^{High}$, another marker of BCSCs, was also examined. After 16 hours exposure to different drugs, the $CD24^{Low}/CD44^{High}$ population in the mammosphere cells was determined by flow cytometry. In comparison with the attached cells the mammosphere population contained a significantly higher percentage of $CD24^{Low}/CD44^{High}$ BCSCs (Fig. 3.11). The percentage of $CD24^{Low}/CD44^{High}$ cells in mammosphere was reduced following 16 hours exposure to DS/Cu and PAC/DS/Cu but not influenced by PAC, DS or Cu (Fig. 3.11).

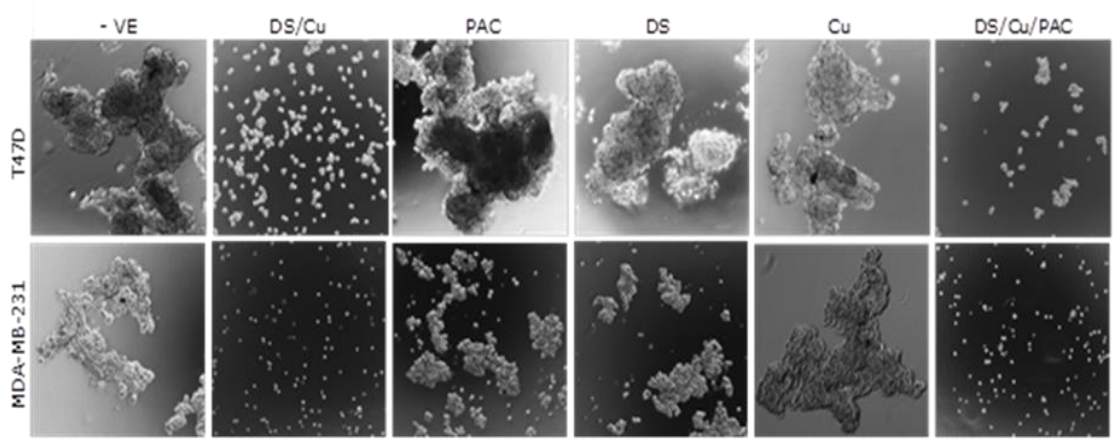


Fig. 3.10 The effect of disulfiram/copper on cancer stem cells in breast cancer cell lines. DS/Cu and PAC/DS/Cu inhibited mammosphere formation. The BC cells were treated with PAC_{40nM}, DS_{250nM}, $Cu_{1\mu M}$, DS/Cu or PAC/DS/Cu for 48 hours and then sub-cultured in drug-free SCM in ultra-low attachment 6-well plates (5,000 cells/well) for 7 days and photographed at × 40 magnification.

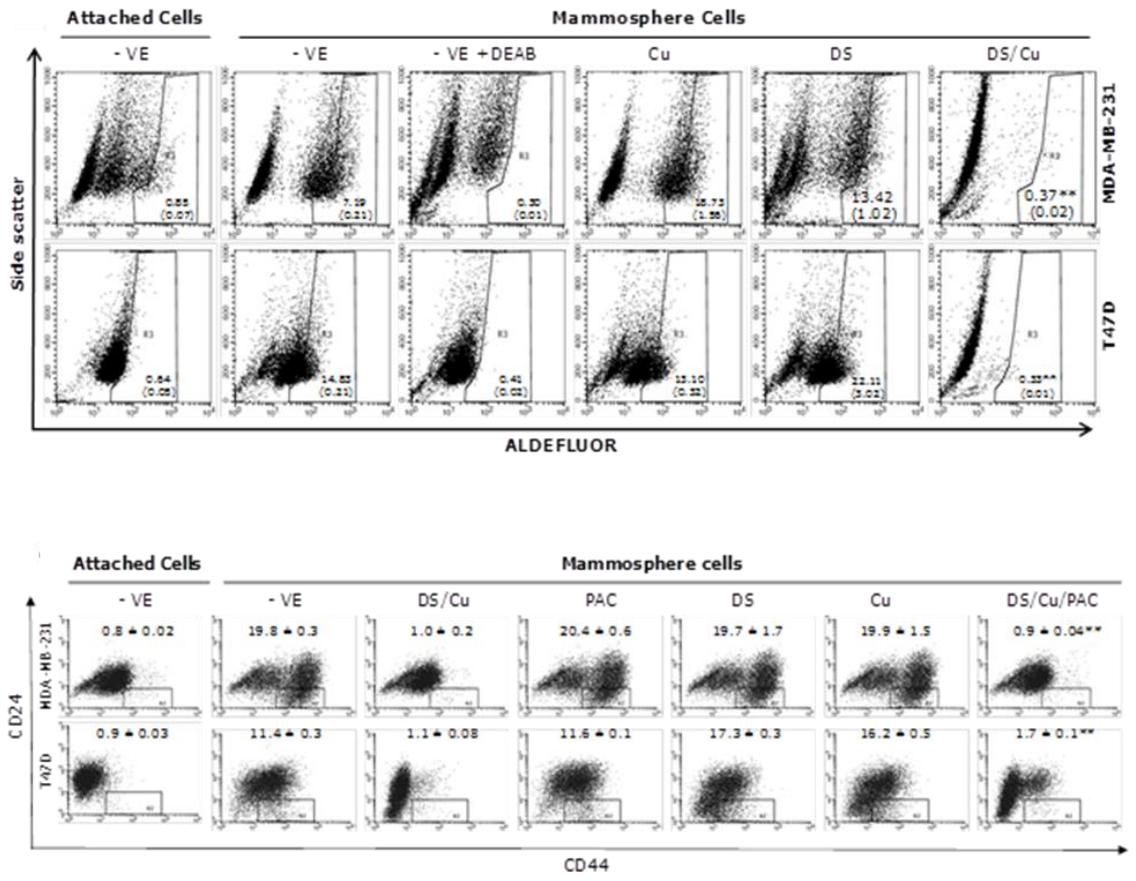


Fig. 3.11 The effect of DS/Cu on CSCs markers in BC cell lines. DS/Cu inhibited ALDH expression in mammospheres. The $ALDH^{+VE}$ population was flow cytometrically determined in mammospheres exposed to drugs ($Cu_{1\mu M}$, $DS_{1\mu M}$ or $DS_{1\mu M}/Cu_{1\mu M}$) for 16 hours. D. DS/Cu abolished $CD24^{Low}/CD44^{High}$ population in mammospheres. The expression of CD24 and CD44 was examined after 16 hours exposure to $Cu_{1\mu M}$, $DS_{1\mu M}$, PAC_{100nM} or DS/Cu/PAC. The inserted numbers in the frame represent percentage of $ALDH^{+VE}$ or $CD24^{Low}/CD44^{High}$ cells (mean ± SD from 3 experiments, **p<0.01).

3.4 Discussion

DS is a Food and Drug Administration-approved anti-alcoholism drug used in clinic for over 60 years with extensive availability of pre-clinical and clinical safety data (Eneanya *et al*., 1981). The study in this chapter

demonstrates that DS is highly cytotoxic to BC cell lines in a Cu-dependent manner. DS can chelate Cu to form a DS/Cu complex. Cu is a crucial trace element that plays a critical role in the redox chemistry, growth and development of every living organism (Turnlund *et al*, 1998). It is also an indispensable catalytic and structural cofactor that drives a wide array of important biochemical processes. In the body Cu exists in both oxidation states, oxidized Cu (II) and reduced Cu (I). The major biological molecules functions of Cu is to react directly with molecular oxygen to produce free radicals. There is a long history of trying to use Cu for cancer therapeutics (Gupte *et al*., 2009; Hieger *et al*, 1926). The successful animal experiment was published almost 9 decades ago. Since then, no further report has been published. Based on our data, single Cu treatment didn't show any cytotoxicity to the T47D and MCF7 cell lines in the MTT assay. The intracellular transport of Cu is still one of the major hurdles for its clinical efficacy due to the complicated environment in the cancer cells. Ctr1 is a Cu transporter, responsible for the transport of Cu into cells (Kim *et al*., 2008). The cellular intake of Cu is tightly controlled by Ctr1. In addition, cancer cells have been reported to show elevated Cu level resulting in higher ROS levels, whereas high Cu is the instinctive characteristic of cancer cells (Kim *et al*., 2008). Due to the intrinsic antiapoptotic mechanisms, no apoptosis is induced in cancer cells.

A derivative of DS, N,N-diethyldithiocarbamate (deDTC), binds to Cu forming a $Cu(deDTC)_2$ complex which improves the intracellular trafficking of Cu and this is probably responsible for DS induced apoptosis (Cen *et al*., 2004). Furthermore, DS can also penetrate into cancer cells to chelate free intracellular Cu to form $Cu(deDTC)_2$. In comparison with normal tissues, many cancers including BC possess higher levels of Cu (2-3 fold) (Margalioth *et al*., 1983; Rizk *et al.*, 1984), which may enable DS to target cancer cells selectively (Bodenner *et al*., 1986; Chen *et al*., 2006; Evans *et al*., 1982; Hacker *et al*., 1982; Iljin *et al*., 2009). Similar results of high level Cu have been found in the cancer cell lines as well. In line with a previous report (Wickstrom *et al*., 2007), a biphasic cytotoxic effect of DS was observed in BC cell lines tested in complete medium without Cu supplement. BC cells were killed at low concentration but revived at higher DS concentrations (~ 10 μM). The mechanism of the biphasic effect is still not very clear. A degraded product of DS may compete trace amounts of Cu, block formation of $Cu(deDTC)_2$ and inhibit transport of Cu into cells (Cen *et al*., 2004).

We have previously reported that DS enhances the cytotoxicity of 5-FU and dFdC in colon and BC cell lines (Guo *et al*., 2009; Wang *et al*., 2003). In this study, we demonstrated the synergistic cytotoxic effect of DS/Cu and PAC on BC cell lines.

Previous studies indicate that in combination with Cu, DS induces ROS activity in melanoma cell lines (Cen *et al*., 2002; Morrison *et al*., 2010). The recent study from Dou's group demonstrates that gold-dithiocarbamato complexes strongly induce ROS and inhibit proteasome activity in BC cells (Zhang *et al*., 2010b). In consistence with these results, our study showed that DS/Cu induced ROS activity which was responsible for DS/Cu induced cytotoxicity in BC cell lines. ROS is not the only target for the cytotoxicity of BC cells. The ROS-induced apoptosis is commonly mediated by the persistent activation of JNK and p38 MAPK pathways (Junttila *et al*., 2008). In our study, I have detected different time points for the treatment of DS/Cu in BC cell lines, the results showed that both JNK and p38 pathways were persistently (over 24 hours) activated (phosphorylation of c-Jun and p38) by DS/Cu and blocked by NAC. JNK and p38 inhibitors reduced cytotoxicity of DS/Cu, although to a lesser degree than ROS inhibition. Therefore ROS-activated JNK and p38 pathways were, at least partially, responsible for ROS-induced apoptosis. The persistent activation of JNK and p38 induces apoptosis via mitochondrial apoptotic pathways (Junttila *et al*., 2008). It is very possible that mitochondrial damage can be detected in BC cells of DS/Cu treatment. The DS/Cu induced apoptosis was confirmed by DNA content and PARP protein cleavage. The expression of BAX and BCL2 proteins was induced and suppressed by DS/Cu respectively leading to increased BAX/BCL2

ratio. The elevated BAX/BCL2 ratio indicated that the intrinsic apoptotic pathway may be involved in DS/Cu induced apoptosis.

Due to the high proliferative rate and energy requirement, cancer cells are under higher ROS stress than their normal counterparts. High levels of ROS can damage DNA, mitochondrial inner membrane and membrane phospholipids leading to apoptosis (Gupte *et al*., 2009). However ROS also activate a wide range of anti-apoptotic factors. The effect of ROS on cancer cells depends on the balance between ROS-induced pro- and anti-apoptotic factors. Further inducing ROS can result in cells undergoing apoptosis. JNK and p38 are the major pathways of MAPK responsible for ROS induced apoptosis. From our results, DS significantly induced ROS activity in BC cell lines. The DS/Cu induced ROS activity in the BC cell lines was reversed by the addition of NAC, a ROS inhibitor. The expression of phosphorylated JNK, c-Jun and total c-Jun was induced by DS/Cu. Meanwhile, high levels of phosphorylated p38 were also detected. ROS have emerged as bridging molecules mediating the crosstalk between NFκB and JNK pathway. Therefore, high ROS is triggered by its inducer DS/Cu thereby actives JNK and p38 pathway to regulate NFκB. NFκB is one of the most important ROS-induced anti-apoptotic factors (Gloire *et al*., 2006). The activation of NFκB in turn inhibits ROS and JNK, p38 activation and ultimately inhibits ROS-induced apoptosis. Consistent with

previous publications (Guo *et al*., 2009; Wang *et al*., 2003), our data indicated that DS/Cu inhibited both binding activity and transcriptional activity of NFκB, and ultimately induced the cells undergo apoptosis. To date, these results indicate that DS/Cu may induce apoptosis of BC cells by simultaneously inducing ROS generation and inhibiting ROS-NFκB pathway.

It has been widely accepted that CSCs are responsible for tumour recurrence and may display significant resistance to different cytotoxic drugs (Liu *et al*., 2010). The characteristics that define CSCs include self-renewal, clonogenic and tumorigenic potential, and transient quiescence, which are believed to contribute to CSCs resistance to conventional therapies. In order to test the effect of DS/Cu on the regeneration of minimal-residual-cancer-cells, I used clonogenic assay, a gold measure to detect the cell 'reproductive death' after cytotoxic agent treatments (Franken *et al*., 2006). In contrast to the moderate inhibiting effect of PAC, DS and Cu on clonogenicity of BC cells, the colony formation was significantly reduced or completely eradicated by DS/Cu and PAC/DS/Cu respectively. These results indicated that a small population of BC cells may be responsible for regeneration of cancer mass after PAC treatment. However, DS/Cu or PAC in combination with DS/Cu can kill these stubborn cancer cells. DS/Cu reverses cancer cell chemoresistance induced

by a wide range of different mechanisms (Guo *et al*., 2009; Wang *et al*., 2004; Wang *et al*., 2001; Wang *et al*., 2003). From the results of the effect of DS/Cu on clonogenicity of BC cell lines, we further examined the effect of DS/Cu on BCSCs.

The stem cell culture system was developed by Dontu (Dontu *et al*., 2003). The cells were cultured in serum-free condition at a non-adherent system. The stem cells can survive and proliferate in this system and form grape-like clusters, which are named as 'mammospheres'. The mammospheres consist of enriched stem cell population, which is capable of differentiating along multiples lineages (Dontu *et al*., 2003). The mammosphere culture system has now been used to identify and enrich the putative stem cells in BC cell lines. Here, we cultured mammosphere of MDA-MB-231 and T47D to examine the effect of different treatments on CSCs population. The mammosphere forming ability in both cell lines was completely blocked by exposure to DS/Cu for 48 hours. Furthermore, we determined the targeting effect of DS/Cu on CSCs and the BCSCs markers in drug-treated mammosphere cells. It has been widely accepted that high ALDH activity can be detected in CSCs (Ginestier *et al*., 2007). This is not only a marker but also responsible for chemo- and radio resistance in BC cell lines MDA-MB-231 and MDA-MB-468 (Croker *et al*., 2012). ALDH1A1 has been identified as a functional marker of several different types of CSCs

including BCSCs (Alison *et al*., 2010; Ginestier *et al*., 2007). Therefore ALDHs may be redundantly expressed in different cancer types, and targeting one isoform may not be sufficiently for CSCs targeting. DS is a strong inhibitor for both cytosol and mitochondrial ALDHs (Eneanya *et al*., 1981; Lam *et al*., 1997). My study is the first report of using DS to target BCSCs. The $ALDH^{+VE}$ population in BCSCs was significantly inhibited by DS/Cu. The ability of BC cell lines to form mammospheres was completely inhibited by exposure to DS/Cu for 24 hours. The effect of DS/Cu on CSCs was also confirmed by the reduction of the $CD24^{Low}/CD44^{High}$ population. The detailed molecular mechanisms underlying the effect of DS/Cu on BCSCs are unclear. ALDHs detoxify intracellular aldehydes which can form adducts with glutathione, nucleic acids and amino acids leading to cell death (Marchitti *et al*., 2008). The high expression of ALDHs in CSCs may be protective. Mammalian cornea cells contain abundant ALDH playing a critical role in scavenging ROS and reducing UV-induced oxidative stress (Estey *et al*., 2007). ALDH deficiency in the central nervous system is associated with progressive neurodegeneration (Marchitti *et al*., 2007). Inhibition of NFκB pathway and induction of ROS results in reduction of stem-like properties in CSCs derived from pancreatic cancer and leukemia (Greten *et al*., 2004; Jin *et al*., 2010; Rausch *et al*., 2010). DS/Cu may target BCSCs by simultaneously inhibiting NFκB and activating ROS activity.

In conclusion, DS/Cu inhibited BCSCs and enhanced cytotoxicity of PAC in BC cell lines. This may be caused by simultaneous induction of ROS and inhibition of NFκB.

4. Disulfiram reverses chemoresistance in an acquired paclitaxel resistant triple negative breast cancer cell line

4.1 Introduction

Triple negative breast cancer (TNBC), an aggressive variant of BC accounting for approximately 15-20% of all BC cases (Carey *et al*., 2006), is characterized by tumours that do not express the ER, PR and HER2 (Bauer *et al*., 2007). Globally, approximately 170,000 cases of TNBC phenotype are detected in the estimated 1 million BC incidences identified annually (Anders *et al*., 2009). Approximately 80% of TNBC cases are classified as basal-like and another minority subtype of TNBC, termed claudin-low, which are uniquely characterized by low to absent expression of differentiated luminal cell surface markers but enrichment of cancer stem cell-like features (Prat *et al*., 2010). In general, TNBC patients are at high risk of early relapse with a sharp decline in survival outcomes during

the first 3 to 5 years after diagnosis (Cheang *et al*., 2008; Liedtke *et al*., 2008; Dent *et al*., 2007). The best median survival period of advanced TNBC is 12 months, much shorter than the duration of survival seen in other subtypes of advanced BC. TNBC has a poorer prognosis than other types of invasive BC, the claudin-low subtype of TNBC has an even poorer prognosis with less of a response to chemotherapy than other basal-like BC. In clinic, general characteristic of TNBC are including present as interval cancer, high risk of early recurrence, rapid progression from the onset of metastasis to death, and highly chemosensitive. The molecular features of TNBC have been described that elevated mitotic activity, amplified Ki67 index, marked cellular pleomorphism, high nuclear cytoplasmic ratio, scant stromal content, central necrosis or central a cellular zones, multiple apoptotic cells, pushing margins of invasion and stromal lymphocytic infiltration (Livasy *et al*., 2006; Rakha *et al*., 2006; Fulford *et al*., 2006; Fadare *et al*., 2007). The optimal therapeutic method for TNBC patients is systemic cytotoxic chemotherapy, which is the mainstay treatment of TNBC, because endocrine therapy or trastuzumab (HER2-targeted agents) produce no clinical benefits for the patients (Liedtke *et al*., 2008; Carey *et al*., 2007; Rouzier *et al*., 2005). However, patients with TNBC have poorer outcomes compared with other BC subtypes. Chemoresistance needs to be overcome in the next few years if any significant clinical strides are to be made. In the early stage of TNBC, neoadjuvant and adjuvant therapy is the

main treatment for patients with TNBC at risk of relapse (Goldhirsch *et al*., 2009). However, cytotoxic chemotherapy is recommended for patients with TNBC who develop metastases. Current treatment agents for both stages of TNBC include anthracyclines, taxanes, and platinum agents. Anthracyclines and taxanes are used as prior neoadjuvant or adjuvant regimen depending on the patient characteristics. TNBC with specific DNA-repair defects is sensitive to platinum agents, thus CDDP-based chemotherapy is more efficacious against TNBC patients (Isakoff *et al*., 2010). More recently, PARP inhibitors, epidermal growth factor receptor (EGFR) inhibitors, and anti-angiogenic agents have been proposed as a therapeutic mechanism in TNBC. Moreover, it has been reported that genistein is well recognised as a protective factor against BC and its mechanism of action has proved to suppress the growth of TNBC cell line as well as inhibiting NFκB activation via Notch-1 signalling pathway (Pan *et al*., 2012). Similarly, a study has reported that α-mangostin has the effect on cell cycle alteration and inducing apoptosis in TNBC cell line (Shibata *et al*. 2011). All these data support that more and more potential chemotherapy agents have been discovered and applied in the treatment of patients with TNBC, whereas there are no preferred chemotherapy regimens are being selected with similar considerations as with other BCs.

Therefore, with on-going studies of platinum-based combination therapy, it is important to find a more effective chemotherapy agent to target TNBC.

As discussed above, due to lack of a molecular target to be tackled, there are very few chemotherapeutic agents available for TNBC chemotherapy. CDDP is commonly used in combination with PAC for the treatment of patients with TNBC. PAC is one of candidate's therapeutic agents in chemotherapy of early stage and metastatic TNBC. PAC targets cancer cells mainly by binding to and stabilising microtubules (Schiff *et al*., 1979), arresting cancer cells in G2/M mitotic checkpoint, and subsequently inducing apoptosis via intrinsic apoptotic pathway (Ferlini *et al*., 2009). As with other anticancer drugs, TNBC cells can develop acquired resistance after repeated exposure to PAC. The acquired chemoresistance remains a major hurdle for the PAC-based chemotherapy. The most recognised resistant mechanisms include overexpression of Pgp encoded by the *ABCB1* gene and alterations in microtubule system (Trock *et al*., 1997). The acquired PAC resistance can also be introduced by mutations in tubulin, which modulate the binding affinity of PAC to microtubules. The following molecular mechanisms are also related to PAC resistance e.g. HER2 overexpression (Knuefermann *et al*., 2003) altered apoptotic and molecular signalling pathways (Takahashi *et al*., 2005; Chabalier *et al*., 2006; Huang *et al*., 1997). Chemotherapy would benefit from identifying

new compounds to target alternative chemoresistant pathways and/or sensitise cancer cells to classical anticancer drugs.

CDDP is a small molecule platinum compound that was first found to inhibit the growth of *Escherichia coli* and later used to treat testicular and bladder cancer (Rosenberg *et al*., 1973). CDDP is employed for the treatment of a wide range of solid malignancies, including testicular, ovarian, head and neck, colorectal, bladder, lung and BCs. CDDP showed a high level and broad spectrum of anticancer activity via multiple mechanisms. The most prominent mode of action involves the generation of DNA lesions followed by the activation of the DNA damage response and the induction of mitochondrial apoptosis, and this effect is related to inhibition of DNA synthesis and repair that might result in cell cycle arrest at the G1, S, or G2-M phase, therefore apoptosis will be induced (Desoize *et al*., 2002). Interestingly, it has been reported that CDDP also induces ROS to trigger cell death *in vitro* and *in vivo*. The formation of ROS depends on the concentration of CDDP and the duration of exposure (Brozovic *et al*., 2010). Further study on the cytotoxicity of CDDP shows that two pathways may contribute to CDDP-induced apoptosis *in vitro*. One involves the tumour-suppressor protein p53, and the other one is mediated by the p53-related protein p73 (Jordan *et al*., 2000). Due to the sensitivity of CDDP is p53 related in cancer, CDDP-inducible p53 gene therapy might

enhance the effectiveness of commonly used chemotherapeutic agents, leading to a promising treatment for cancers (Wang *et al*., 2006).

The first exciting clinical trial of CDDP in BC patients without previous chemotherapy was reported by Kolaric and Roth (1983) with a dose of 30 mg m^{-2} I.V. daily for 4 days every 3 weeks (Kolaric *et al*., 1983). After this, an accumulating number of studies suggest CDDP can be used as the first line anticancer drug in BC, especially in TNBC. However, the single drug therapy may induce chemoresistance. In order to increase the patient's survival periods, CDDP is currently used in combination with other anticancer drugs such as dFdC or PAC in chemotherapy, and also in Phase I to III clinical trials. Acquired drug resistance occurs when tumour cells do not respond to treatment with anticancer drugs including CDDP. Several CDDP resistance mechanisms are as follows, (a) decreased intracellular drug accumulation and/or increased drug efflux; (b) drug inactivation by increased levels of cellular thiols; (c) evasion of apoptosis by increased nucleotide excision-repair activity and decreased mismatch-repair activity; (d) alterations in drug target (Sedletska *et al*., 2005; Brabec *et al*., 2005; Kartalou *et al*., 2001). Due to the unwanted resistance of CDDP, patients receiving CDDP experience severe side effects that limit the dose, which can be administered. The side effects of CDDP include nausea and vomiting, myelosuppresion and immunosuppression. More specific side

effects include damage to kidneys (nephrotoxicity), peripheral nerves (neurotoxicity) and the inner ear (ototoxicity) (Shah *et al*., 2009; Gunes *et al*., 2009; Florea *et al*., 2006). CDDP has proven to be one of the more efficient anticancer chemotherapeutic agents in the fight against several solid cancers. Unfortunately, cancer cells either intrinsically resistant or relatively rapidly become resistant to CDDP, leading to relapse and therapeutic failure. Therefore, more research is necessary in order to improve anticancer treatments and to decrease toxic effects.

The MDA-MB-231 cell line originated from an epithelial adenocarcinoma of the mammary gland, which was isolated from a pleural effusion of a 54 year old Caucasian female in 1971 (Zhang *et al*., 1991). This cell line is one of the commonly used types for screening of new anti-neoplastic drugs by National Cancer Institute. MDA-MB-231 cells possess an 'epithelial like' appearance as they are adherent, invasive and poorly differentiated. Furthermore, they also behave aggressively, are metastatic in nature and have been found to express neither estrogen, progesterone nor HER2 receptor, apart from the presence of basal expression of epidermal growth factor receptor (EGFR). Hence, MDA-MB-231 is TNBC cell lines. In addition, these cells also acquire p53 mutation and strong Ki67 expression, which is an important marker for cell proliferation (Neve *et al*. 2006; Keam *et al*. 2011).

It has been suggested that human BC contains a small population of CSCs, which can be detected by the expression of stem cell markers (ALDHs, $CD24^{Low}/CD44^{High}$) and activation of embryonic related pathways (Sox2, Oct4, Nanog) (Marques *et al*., 2010). BCSCs are slow cycling and quiescent population expressing high levels of Pgp (Dean *et al*., 2009). The cancer cells with CSC phenotypes are highly resistant to a variety of conventional anticancer drugs and responsible for cancer recurrence. Targeting CSCs may improve the outcomes of cancer chemotherapy (Clevers *et al*., 2011).

DS, a commercially available anti-alcoholism drug, shows anticancer activity *in vitro* and *in vivo*. It also potentiates cyclophosphamide, CDDP and radiation *in vitro* and protects normal cells in kidney, gut and bone marrow *in vivo* whilst increasing the therapeutic index of cytotoxic drugs. The randomized clinical trial indicates that in combination with chemotherapy, ditiocarb, the derivative of DS, significantly improves 5-year overall survival of high risk BC patients. The anticancer activity of DS is Cu dependent. Cu plays a crucial role in redox reactions and triggers generation of ROS in human cells. DS/Cu is a strong ROS inducer and proteasome-NFκB pathway inhibitor. DS specifically inhibits the activity of ALDH, a functional CSCs marker and ROS scavenger. A combination of DS with Cu may target cancer cells by simultaneous modulation of both

ROS and NFκB. DS and its metabolites can also covalently modify cysteine residues within nucleotide binding domain of Pgp and permanently inhibit Pgp activity. This will potentially reverse multidrug resistance.

4.2 Methods

General methods have been described in chapter II, the following are the methodologies specifically used in this study.

4.2.1 Cell lines and reagents

The PAC resistant cell line (MDA-MB-231$_{PAC10}$) was generated from MDA-MB-231 (purchased from ATCC) by being continuously cultured in medium containing PAC (Sigma, Dorset, UK) respectively in a stepwise concentration-increasing procedure. CDDP, DS and copper (II) chloride ($CuCl_2$) were purchased from Sigma.

4.2.2 Grow curves and doubling time analysis

The cells (5×10^3/well) were cultured in 24-well plates in triplicate. The cells were collected by trypsinization and cell numbers in each of three wells were counted every 24 hours for 120 hours. The cell doubling time was calculated using the program from the Doubling Time Online Calculator http://www.doubling-time.com/compute.php.

4.2.3 Cell culture and cytotoxicity analysis

All cell lines were cultured in DMEM (Lonza, Wokingham, UK) supplemented with 10% FCS, 50 units/ml penicillin, and 50 μg/ml streptomycin. The MDA-MB-231$_{PAC10}$ cells were maintained in the medium containing 10 nM of PAC. For *in vitro* cytotoxicity assay, the overnight cultured cells (5,000/well) in 96-well flat-bottomed microtiter plates were exposed to drugs for 72 hours (PAC) or 120 hours (CDDP) and subjected to a standard MTT assay.

4.2.4 Immunofluorescent flow cytometry and confocal microscopy

The expression of Nanog, Oct4 and Sox2 was determined by immunofluoresence flow cytometry and confocal microscopy. For immunocytochemistry confocal microscopy analysis, the cells were grown on a culturing chamber slide (Sigma, Dorset, UK) overnight and fixed by acetone/methanol and permeabilized by 0.1% triton-X100. After being blocked with 3% BSA for 1 hour the cells were stained with primary antibodies (1:50 dilution 5 μg/ml) and FITC-conjugated secondary antibody for 1 hour at RT. The coverslips were mounted on glass slides with VectaShield mounting media containing the nucleic acid stain, 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame,

CA), and examined by laser scanning confocal microscopy using a Zeiss Axiovert 200 microscope and ZEN 2009 software (Carl Zeiss Canada Ltd., Mississauga, CA). For immunofluorescent flow cytometric analysis, the cells were cultured in T25 flasks until 80% confluence and collected by trypsinization. The cells were stained in suspension using the same concentration of antibodies and procedure as immunocytochemistry analysis. The positively stained population was detected using a FACS Calibur flow cytometer with 488 nm blue laser and standard FITC 530/30 nm band pass filter.

4.3 RESULTS

4.3.1 MDA-MB-231$_{PAC10}$ cells are resistant to chemotherapy drugs

The cytotoxic effect of PAC on both sensitive and resistant cell lines was compared by MTT assay (Table. 4.1 and Fig. 4.1A). The MDA-MB-231 cells are sensitive to the cytotoxicity of PAC with an IC_{50}_72h of 8.7nM. In contrast, the MDA-MB-231$_{PAC10}$ cell line is highly resistant to PAC with an IC_{50}_72h over 1000 nM. The cytotoxic effect of CDDP on MDA-MB-231$_{PAC10}$ cell line was also evaluated. It has been demonstrated that MDA-MB-231$_{PAC10}$ cells are also significantly cross-resistant to CDDP (Table. 4.1 and Fig. 4.1B).

Table 4.1 Cytotoxicity of PAC, CDDP and DS to MDA-MB-231 and MDA-MB-231$_{PAC10}$ breast cancer cell lines

	MDA	PAC10
PAC (nM)	8.7 (2.3)	>1000**
CP (nM)	256.7 (26.1)	645.4* (127.3)
DS (nM)	151.9 (12.1)	116.4 (30.0)

The table represents IC_{50} value from three experiments [mean (SD)]. **p<0.01' *p<0.05 (n=3). The cells were treated for 72 hours. DS/Cu: DS in medium supplemented with 1μM $CuCl_2$. CP: cisplatin (CDDP); PAC: paclitaxel; DS: disulfiram.

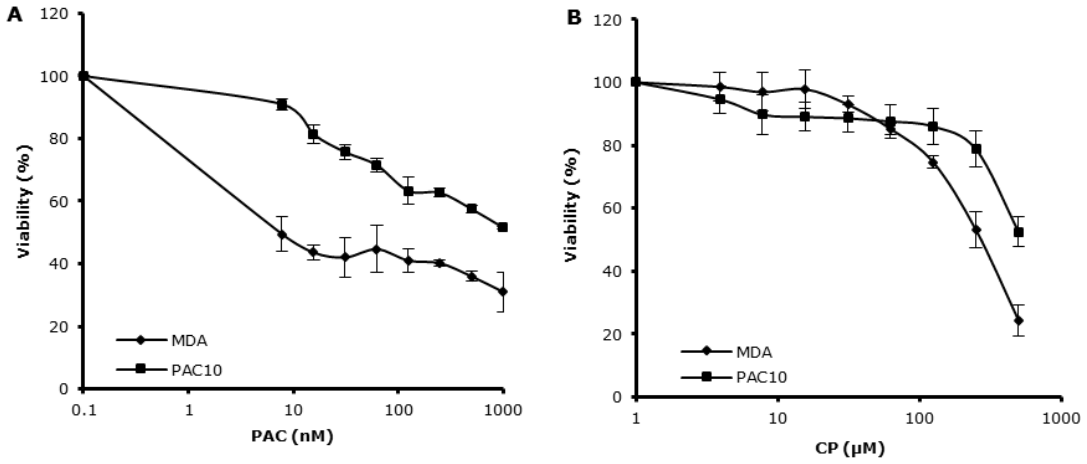


Fig. 4.1 MDA-MB-231$_{PAC10}$ cell line is resistant to PAC and cross-resistant to CDDP. The MDA-MB-231$_{PAC10}$ and MDA-MB-231 cell lines were exposed to PAC (A) and CDDP (B) for 72 and 120 hours respectively.

In line with the MTT data PAC (20 nM) abolished the clonogenicity of the parental cell line but no effect on MDA-MB-231$_{PAC10}$ cells (Fig. 4.2A and B). Due to the slower proliferation rate, the colonies developed from the resistant cell line are smaller than that from the parental cell line.

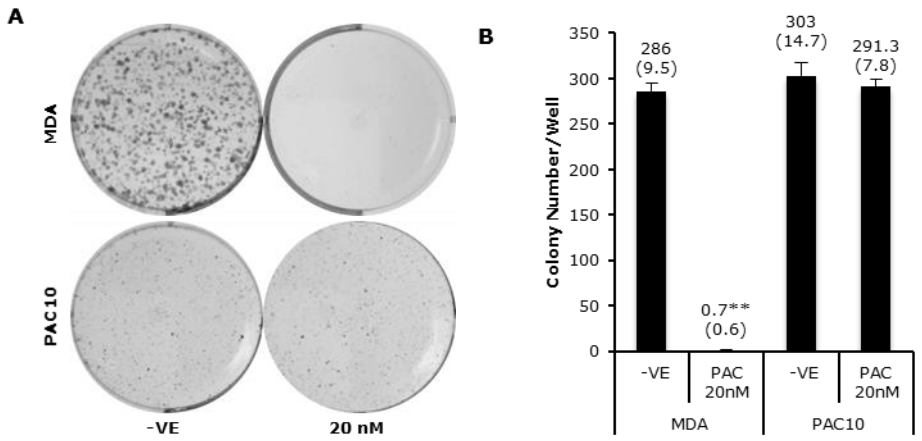


Fig. 4.2 Clonogenic assay of MDA-MB-231$_{PAC10}$ and MDA-MB-231. (A) and (B) The cells were exposed to PAC (20 nM) for 72 hours and then subcultured in drug-free medium at a cell density of 2,500 cells/well in 6-well plates for 10 days. The colony number of clonogenic assay. The colonies with ≥ 50 cells were counted. The figures indicate mean (SD). N=3, **p<0.01.

MDR1 overexpression is the most common mechanism involved in multidrug resistance which includes PAC resistance. High expression of Pgp was detected in the PAC resistant cell line by Western blot (Fig. 4.3A). PAC induces apoptosis mainly via intrinsic apoptotic pathway. Therefore the protein expression status of BAX and BCL2 (two major components involved in intrinsic apoptotic pathway) was examined by Western blot. Fig. 4.3B shows that the MDA-MB-231$_{PAC10}$ cell line expresses significantly higher background levels of BCL2 protein than those in the parental cells. The BCL2/BAX ratio in the resistant cell line is markedly higher than that in the parental cell line.

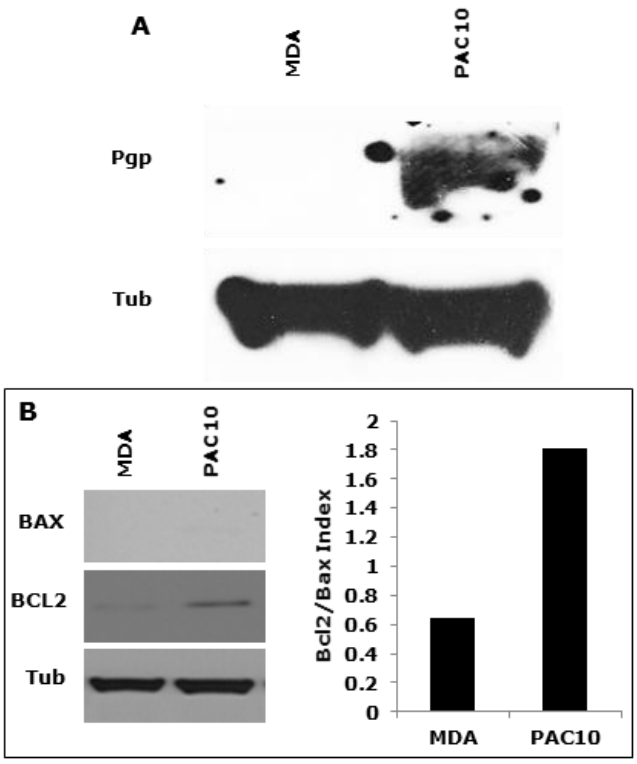


Fig. 4.3 Protein expression detected by Western blotting. Protein expression of Pgp (A), BAX and BCL2 (B). MDA: MDA-MB-231; PAC10: MDA-MB-231$_{PAC10}$

4.3.2 Resistance of MDA-MB-231$_{PAC10}$ cell line to paclitaxel-induced apoptosis

After 72 hours exposed to 20 nM PAC, the phase contrast microscopic images demonstrate apoptotic morphologies (cell blebbing and nuclear condensation and fragmentation) in MDA-MB-231 but not in the MDA-MB-231$_{PAC10}$ resistant cells (Fig. 4.4).

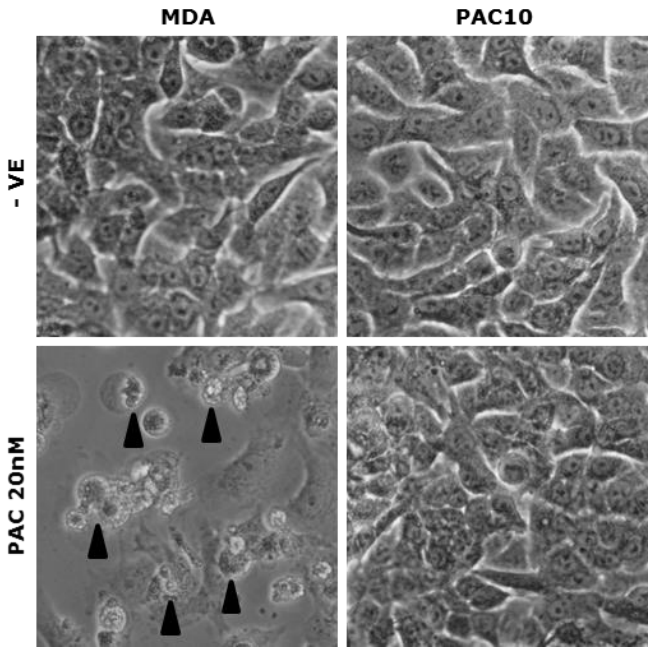


Fig. 4.4 MDA-MB-231$_{PAC10}$ cell line is resistant to PAC-induced apoptosis by morphologies observation. The morphology (× 200 magnification) of parental and resistant cells after 72 hours exposure to PAC (20 nM).

Flow cytometric DNA content analysis manifested that PAC induced a significantly higher ($p<0.01$) apoptotic sub-G1 population (30.4%) in the parental cell line than those in the untreated cells (0.4%) (Fig. 4.5A). PAC (20 nM, 72h) also introduced G2/M-phase blockade leading to an increased G2/M population (untreated: 17.9%, treated: 36.4%; $p<0.01$) and a decreased G0/G1 population (dropped from 64.9% to 15.6%, $p<0.01$) (Fig. 4.5B) in the parental cell line. In contrast, there is no significant effect of PAC on the apoptotic status in the resistant cells. The cell cycle status in MDA-MB-231$_{PAC10}$ cell line is also not affected by PAC exposure (Fig. 4.5C).

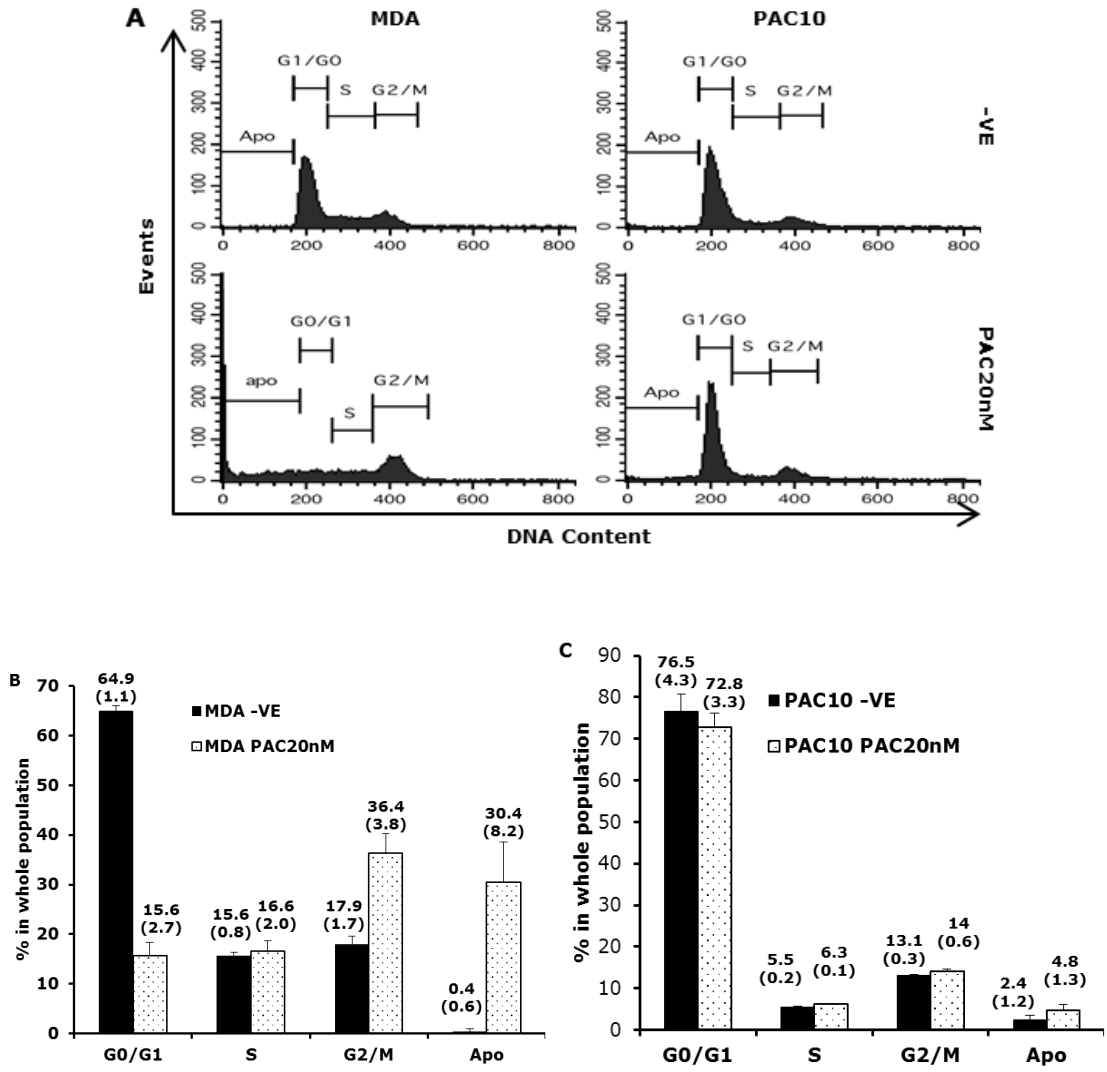


Fig. 4.5 MDA-MB-231$_{PAC10}$ cell line is resistant to PAC-induced apoptosis detected by flow cytometric DNA analysis. (A) Histogram of flow cytometric DNA content analysis. The effect of PAC (20 nM, 72 hours) on cell cycle parameters in MDA-MB-231 (B) and MDA-MB-231$_{PAC10}$ (C) cell lines. The figures represent mean (SD) from three experiments.

As mentioned above, BAX and BCL2 are two golden components involved in intrinsic apoptotic pathway, thus PAC inducible apoptosis was examined by Western blot. Fig. 4.6 shows that PAC exposure induces BAX expression leading to high BAX/BCL2 ratio in the parental cells but no effect on the resistant cells.

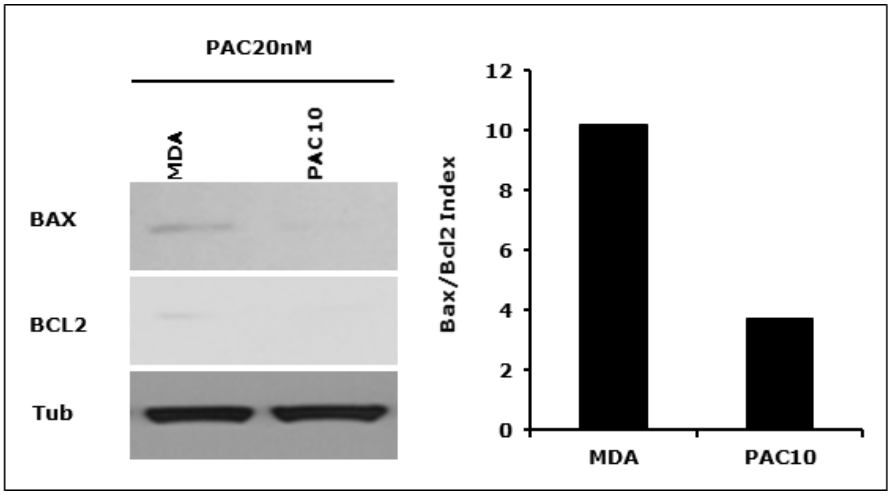


Fig. 4.6 BAX and BCL2 protein expression. Western blotting analysis of BAX and BCL2 protein expression in MDA-MB-231 and MDA-MB-231$_{PAC10}$ cell lines after exposure to PAC (20nM) for 72 hours.

4.3.3 MDA-MB-231$_{PAC10}$ has longer doubling time

In the cell culture, the drug resistant MDA-MB-231$_{PAC10}$ cells grow markedly slower than MDA-MB-231 cells. Therefore we compared the doubling time and cell cycle parameters in these two cell lines. Fig. 4.7 shows the growth curves of both cell lines. The doubling time of MDA-MB-231$_{PAC10}$ cells (64.9h) is significantly longer than that of the sensitive cells (31.7h) ($p<0.01$).

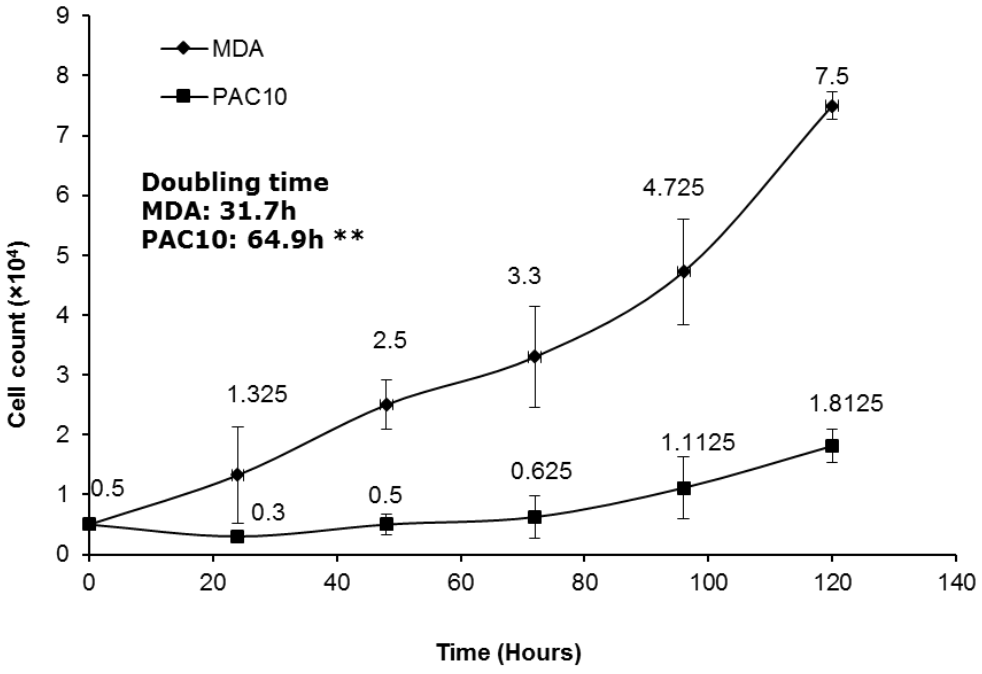


Fig. 4.7 Growth curves of MDA-MB-231 and MDA-MB-231$_{PAC10}$. MDA-MB-231$_{PAC10}$ cell line has longer doubling time. MDA: MDA-MB-231, PAC10: MDA-MB-231$_{PAC10}$. The figures represent the mean of three experiments. **p<0.01.

Further cell cycle study on both cell lines using flow cytometry analysis (Fig. 4.8) shows that in comparison with the parental cell line, the MDA-MB-231$_{PAC10}$ cells have significantly higher G0/G1 and lower S-phase population.

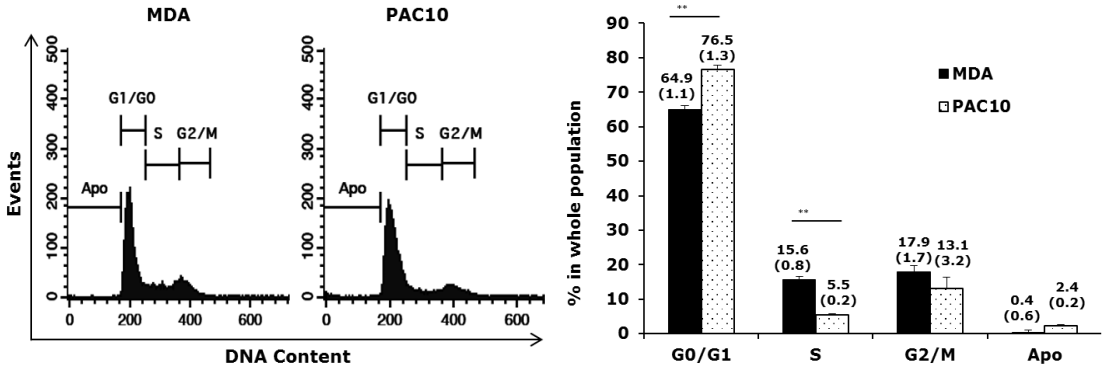


Fig. 4.8 Cell cycle status of MDA-MB-231 and MDA-MB-231$_{PAC10}$ cell lines respectively. The figures represent mean (SD) of three experiments. **p<0.01.

The expression levels of cell cycle determinant proteins were examined by Western blot. Fig. 4.9 shows the Western blotting image and relative band density analysed by ImageJ program. Relative density index = (Target protein/Tubulin) x 100. The expression of p21 is tightly controlled by p53 (Lazzarini *et al*., 2008), and p21 protein mediates the p53-dependent cell cycle G1 phase arrest in response to chemotherapy agents. Therefore, the expression of p21 and p53 was examined (Woods *et al*., 1997). The results showed p21 is markedly overexpressed in the resistant cell line, but p53 is moderately up-regulated, so are cyclin D1 and cyclin E.

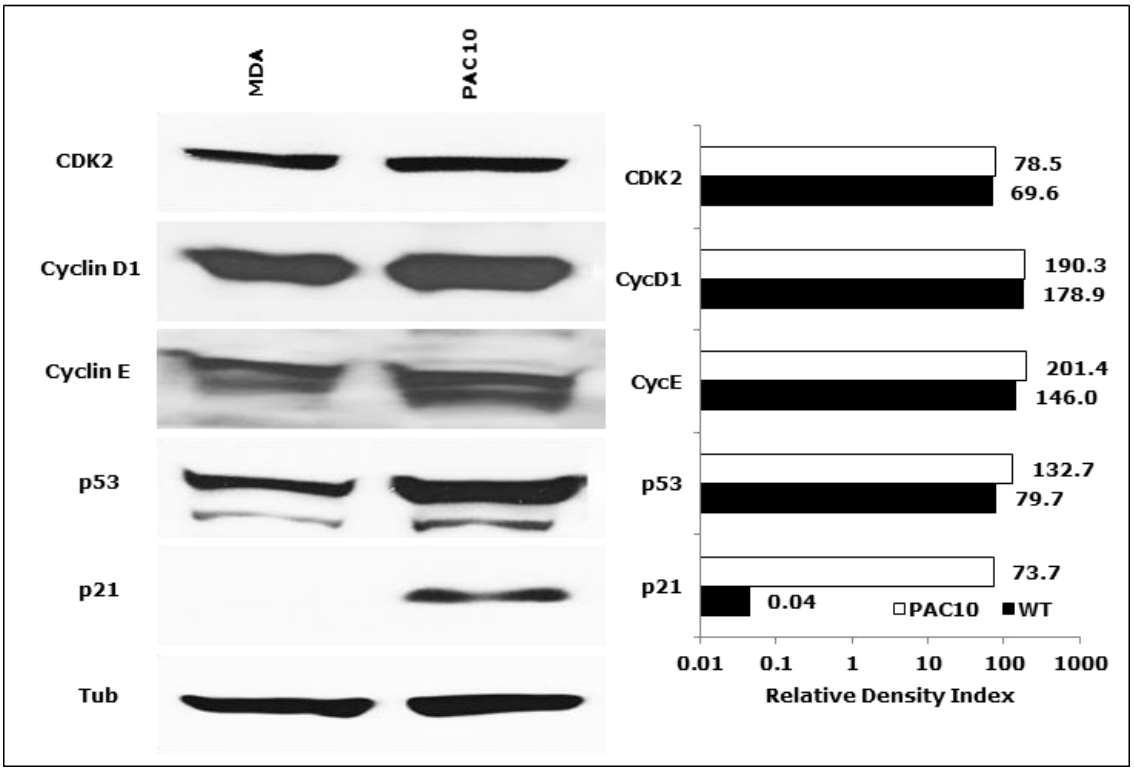


Fig. 4.9 Expression of cell cycle protein. Western blotting analysis of cell cycle related proteins in MDA-MB-231 and MDA-MB-231$_{PAC10}$. The bar chart on the right represents relative density index of the bands.

4.3.4 MDA-MB-231$_{PAC10}$ cells demonstrate cancer stem cells characteristics

It has been widely accepted that CSCs are responsible for chemo- and radio-resistance. The resistant cell line is slow cycling with high expression of p21 protein and expresses high levels of Pgp which are the common features in CSCs. Therefore we examined CSC markers in the resistant and parental cell lines. High ALDH activity is a functional marker of CSCs derived from different cancer types including BC (Ginestier *et al*., 2007). Fig. 4.10 shows that in comparison with the parental cells the MDA-MB-

231_{PAC10} cell line possesses higher $ALDH^{+}$ population which also express higher levels of embryonic stem cell markers (Oct4, Sox2 and Nanog).

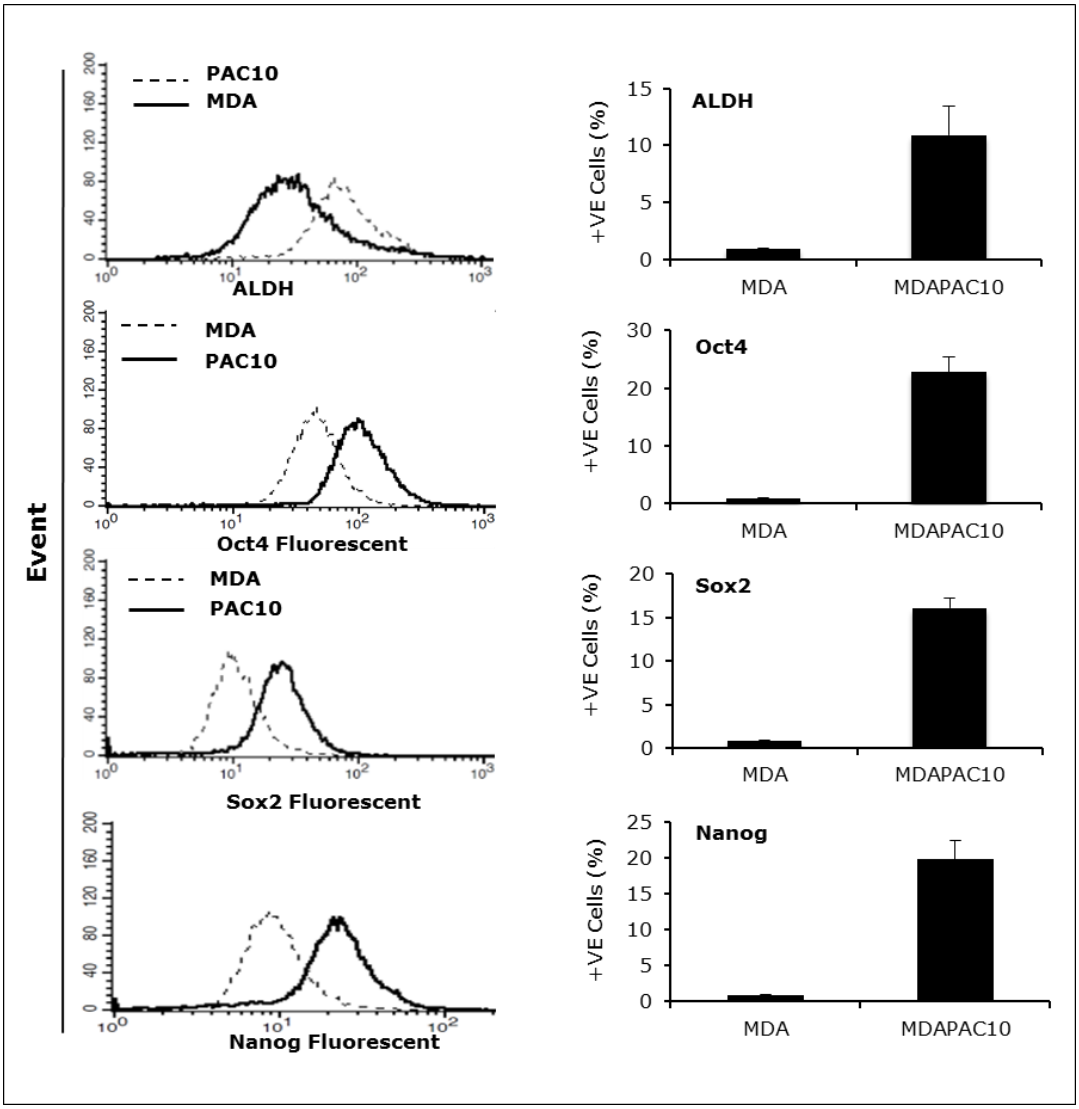


Fig. 4.10 Cancer stem cell markers were expressed in MDA-MB-231_{PAC10} cell line detected by Flow cytometry. Flow cytometric analysis of ALDH activity and Oct4, Sox2 and Nanog protein expression levels.

In order to further confirm the CSC marker existing in MDA-MB-231_{PAC10} cell line, immunofluorescence confocal microscopy has been used in my

study. Fig. 4.11 shows high expression of Oct4 and Sox2 in the PAC resistant cell line.

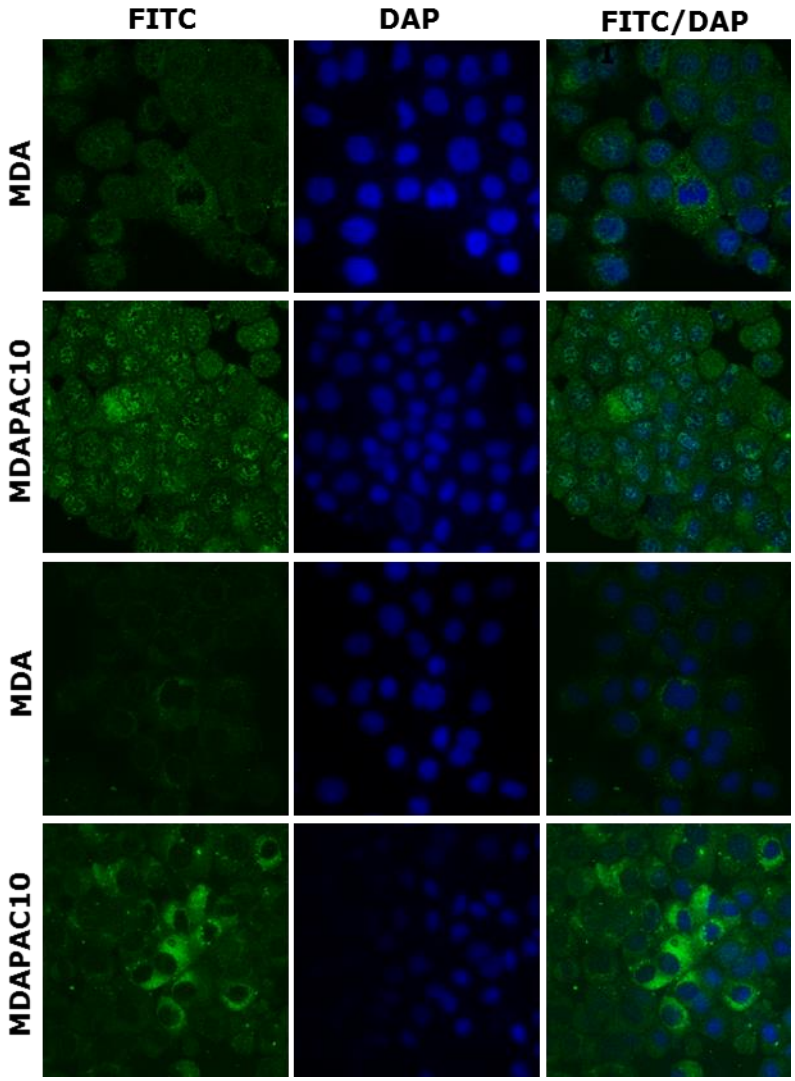


Fig. 4.11 Cancer stem cell markers were expressed in MDA-MB-231PAC10 cell line detected by confocal microscopy. High expression of Oct4 and Sox2 was detected in MDA-MB-231$_{PAC10}$ cells by immunofluorescence confocal microscopy.

The overexpression of Oct4 and Sox2 protein was also detected by Western blotting assay (Fig. 4.12). The expression of NFκB and HIF2α protein was also examined by Western blotting analysis because emerging evidence

indicates that hypoxia and NFκB are responsible for maintaining stemness in CSCs (Wang *et al*., 2004). In comparison with the parental cell line, higher levels of HIF2α and NFκB p65 protein were detected in the nuclear protein of MDA-MB-231$_{PAC10}$ cells. The expression levels of cytoplasmic NFκB p65 in the resistant and parental cell lines are comparable (Fig. 4.12).

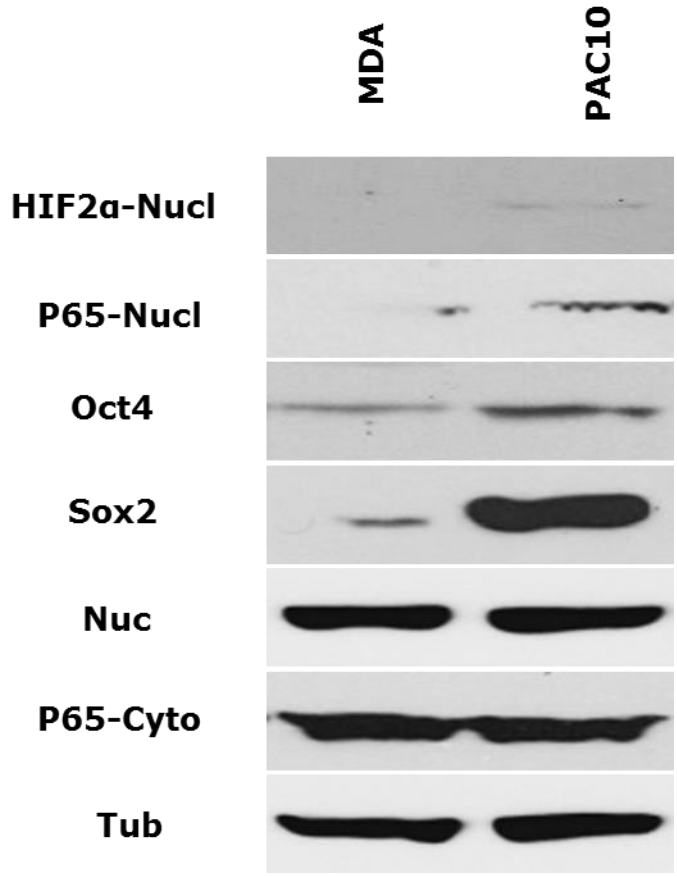
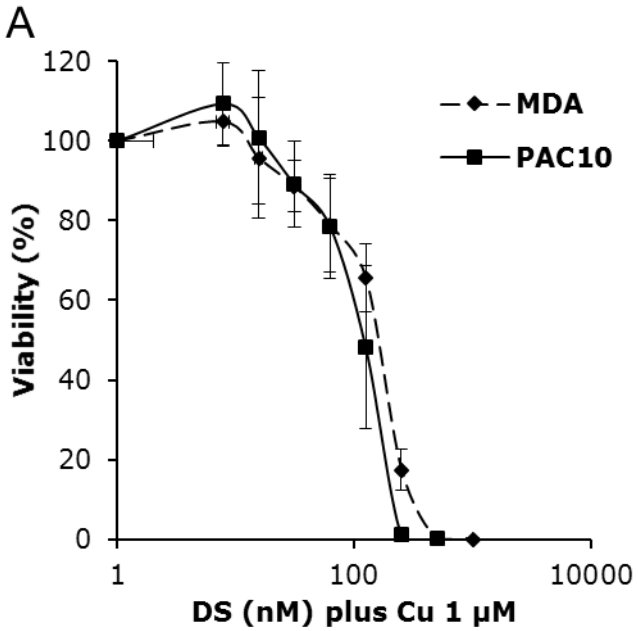


Fig. 4.12 Protein expression of cancer stem cell markers. High expression of HIF2α, NFκBp65, Sox2 and Oct4 protein was detected in nucleus of MDA-MB-231$_{PAC10}$ cell line. Nucl, nucleus; Nuc, nucleolin; Tub, α-tubulin.

4.3.5 Disulfiram is highly cytotoxic in MDA-MB-231$_{PAC10}$ cells and reverses paclitaxel resistance

Our previous studies demonstrate that DS is a strong CSCs inhibitor and highly cytotoxic to a wide range of cancer cell lines. In spite of resistance to PAC and CDDP, the sensitivity of MDA-MB-231$_{PAC10}$ cell line to DS is comparable to that of parental cells (Fig. 4.13). The clonogenecity of both parental and resistant cell lines is completely abolished after very short exposure (4 hours) to DS /Cu (Fig. 4.13).



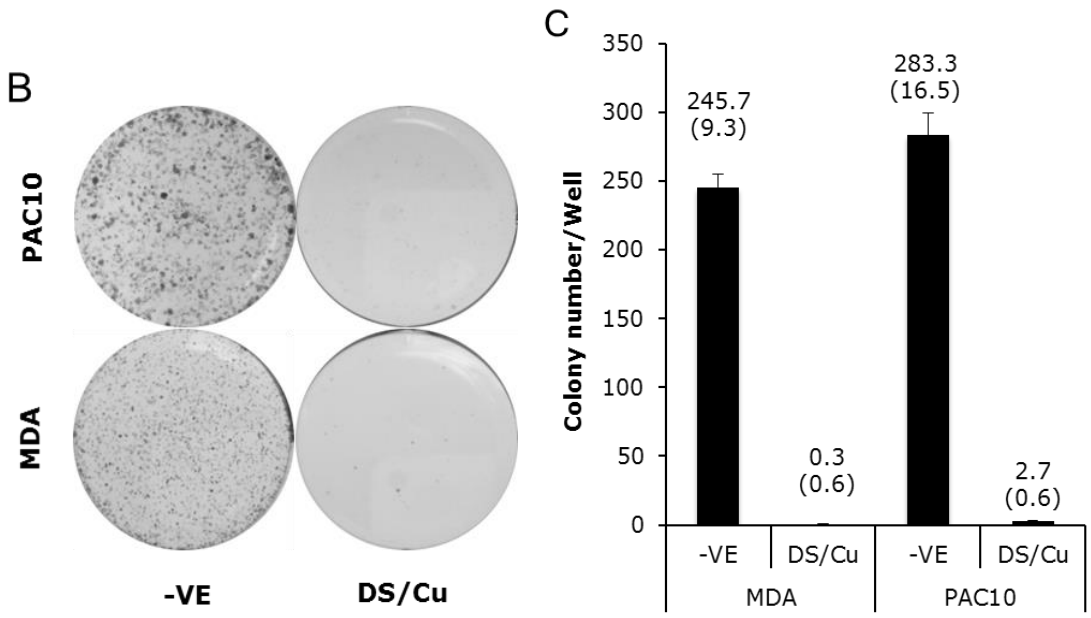


Fig. 4.13 Disulfiram is highly cytotoxic in MDA-MB-231$_{PAC10}$ cell line as well as in MDA-MB-231. (A) MTT cytotoxicity assay. The MDA-MB-231 and MDA-MB-231$_{PAC10}$ cell lines were exposed to different concentrations of DS in combination with 1μM of $CuCl_2$ for 4 hours and then released in drug-free medium for 72 hours. (B) and (C) Clonogenic assay. The cells were exposed to DS 1as plus Cu 1lu for 4 hours and then subcultured in drug-free medium at a cell density of 2,500 cells/well in 6-well plates for 10 days. The colony number of clonogenic assay. The colonies with ≥ 50 cells were counted. The figures indicate mean (SD). N=3.

4.3.6 Disulfiram/copper induces apoptosis in MDA-MB-231$_{PAC10}$ cell line

After 24h exposed to DS/Cu, the phase contrast microscopic images show massive apoptotic cells were detected in MDA-MB-231$_{PAC10}$ (Fig. 4.14A). Flow cytometric DNA content analysis indicated that DS/Cu induced a significantly higher ($p<0.01$) apoptotic sub-G1 population (50%) in resistant cell line than those in the untreated cells (5%) (Fig. 4.14B and C).

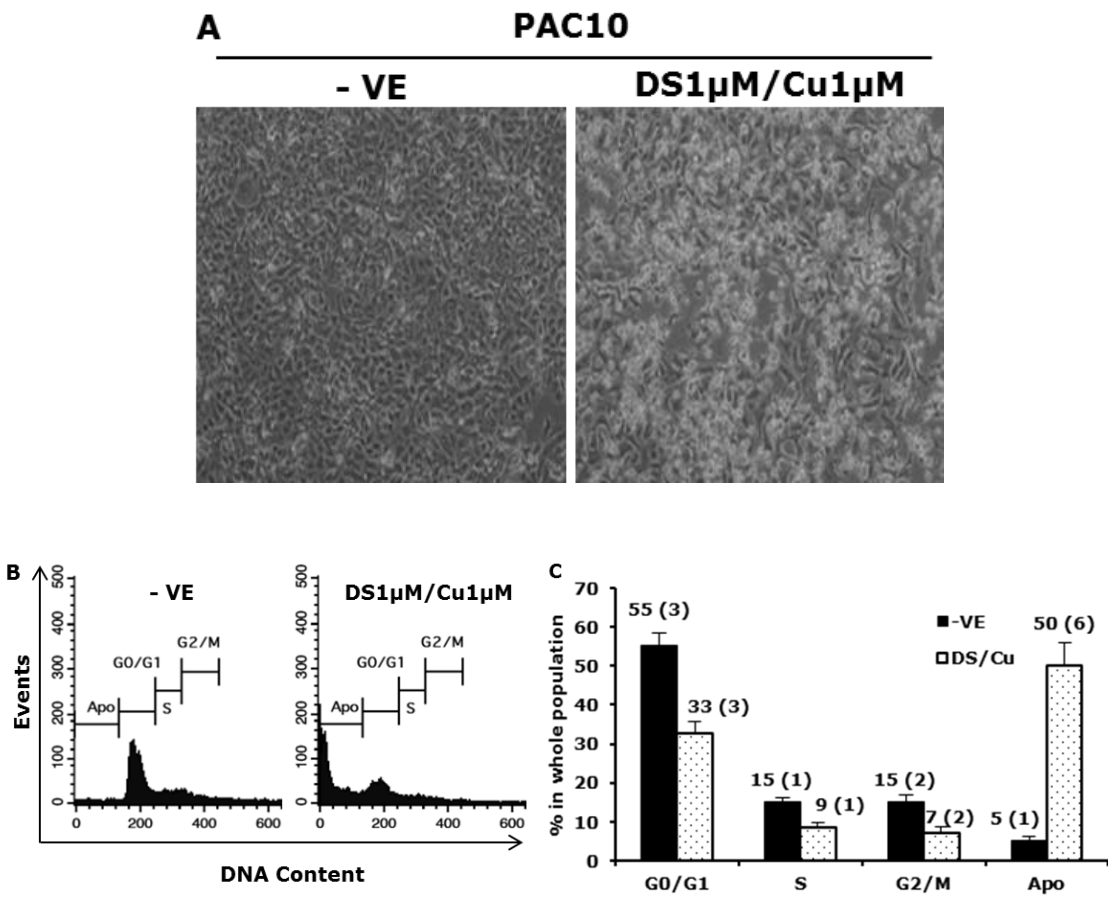


Fig. 4.14 Disulfiram induces apoptosis in MDA-MB-231$_{PAC10}$ cells. (A) The morphology (×100 magnification) of MDA-MB-231$_{PAC10}$ cells after drug exposure. (B) and (C) The influence of DS/Cu on cell cycle parameters in MDA-MB-231$_{PAC10}$ cells. The figures in C represent mean (SD) from three independent experiments. DS $_{1\mu M}$ /Cu $_{1\mu M.}$

DS/Cu inhibits and induces the expression of Bcl2 and BAX in MDA-MB-231$_{PAC10}$ cells respectively leading to significantly increased BAX/BCL2 ratio in the resistant cell line (Fig. 4.15). Although DS is a specific inhibitor of MDR1 activity, the protein expression of Pgp in MDA-MB-231$_{PAC10}$ cell line was not affected by DS/Cu exposure (Fig. 4.15).

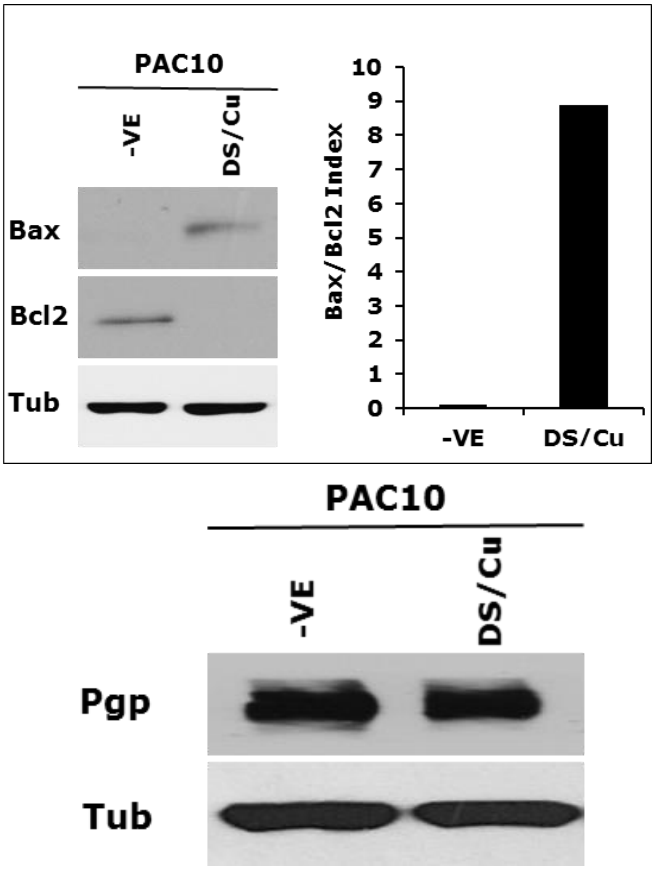


Fig. 4.15 The effect of Disulfiram on apoptosis related protein expression in MDA-MB-231$_{PAC10}$ cells. Western blotting analysis of DS-induced alteration in BAX, BCL2 and Pgp expression in MDA-MB-231$_{PAC10}$ cells. The cells were exposed to DS (1μM) and $CuCl_2$ (1μM) for 4 hours and then released in drug-free medium for 24 hours.

The effect of DS/Cu on cell cycle regulating proteins was analysed by Western blot. Fig. 4.16 shows that DS/Cu induces the expression of p21 and p53 protein but has no effect on CDK2, Cyclin D1 and E expression.

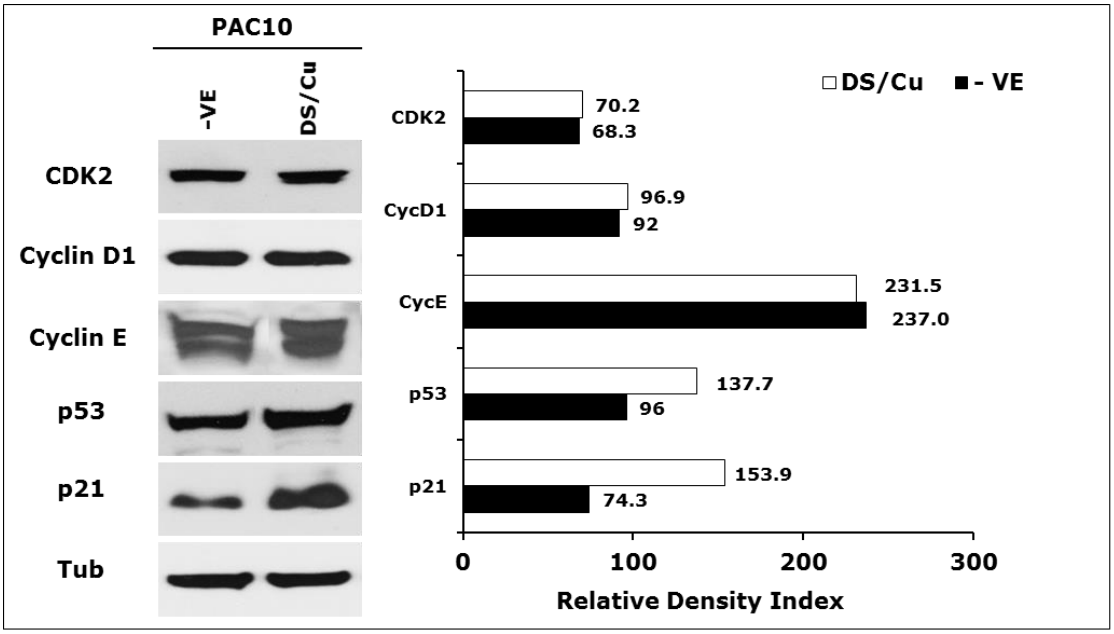


Fig. 4.16 The effect of Disulfiram on cell cycle related protein expression in MDA-MB-231$_{PAC10}$ cells. Western blotting examination of the effect of DS/Cu on cell cycle related protein expression. The cells were exposed to DS (1μM) and $CuCl_2$ (1μM) for 4 hours and then released in drug-free medium for 24 hours.

4.3.7 Disulfiram inhibits the expression of cancer stem cells markers and reverses paclitaxel resistance in MDA-MB-231$_{PAC10}$ cells

Nowadays, CSCs are believed to be one of the key factors involved in chemoresistance (Balic *et al*., 2006). The MDA-MB-231$_{PAC10}$ cell line is composed of high population of CSCs. Furthermore, we examined if DS/Cu inhibits the CSCs in the resistant cell line. The ALDH activity in the resistant cell line is inhibited after exposure to DS/Cu for 4 hours. DS/Cu

also inhibits the expression of Sox2 and Nanog in the resistant cells (Fig. 4.17).

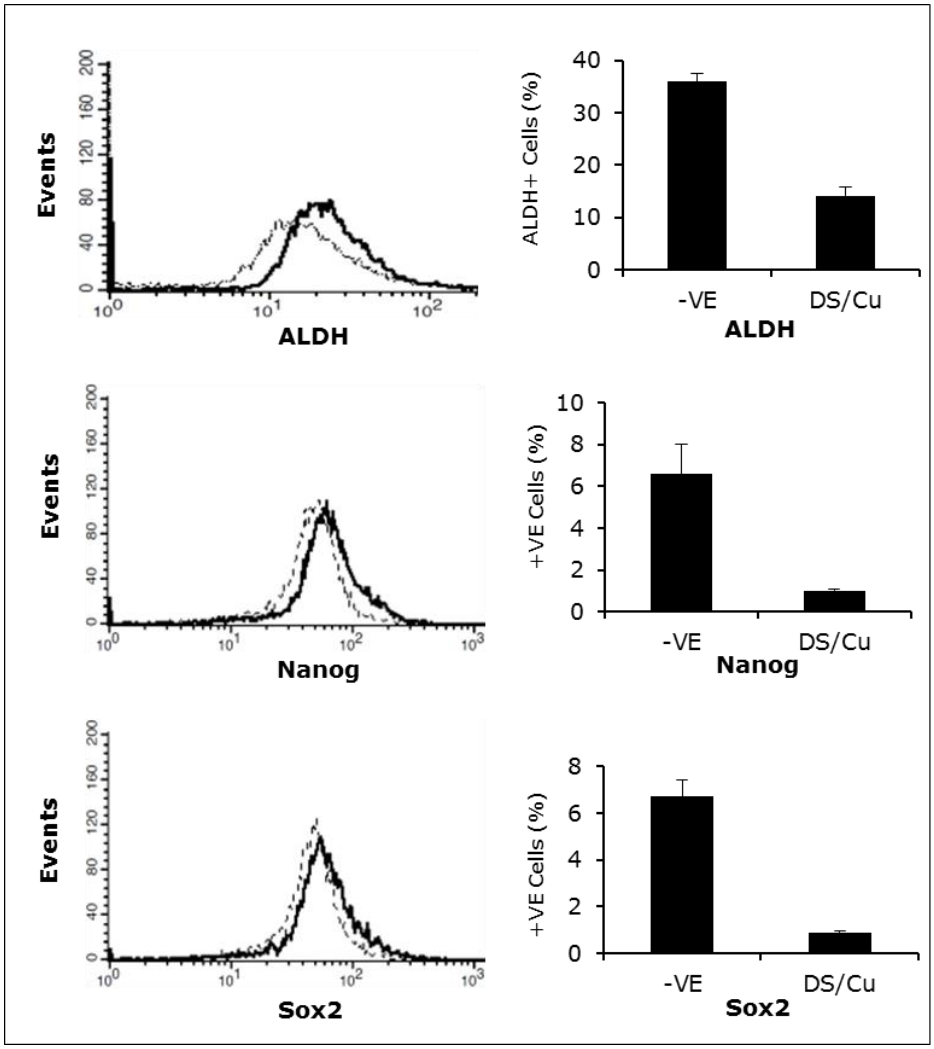


Fig. 4.17 Disulfiram/copper inhibits cancer stem cells markers in MDA-MB-231$_{PAC10}$ cells. DS/Cu inhibits ALDH activity and the expression of Sox2 and Nanog protein in MDA-MB-231$_{PAC10}$ cell line. The cells were exposed to DS (1μM) and $CuCl_2$ (1μM) for 4 hours.

Furthermore, I also examined whether DS/Cu can enhance the cytotoxicity of PAC and reverse PAC resistance in MDA-MB-231$_{PAC10}$ cell line. In combination with DS/Cu, the cytoxicity of PAC in MDA-MB-231$_{PAC10}$ cells is markedly higher than exposure to PAC or DS/Cu alone (Fig. 4.18).

CI-isobologram indicates that the cytotoxicity of DS/Cu + PAC is synergistic in a wide range of concentrations (IC_{20}-IC_{95}).

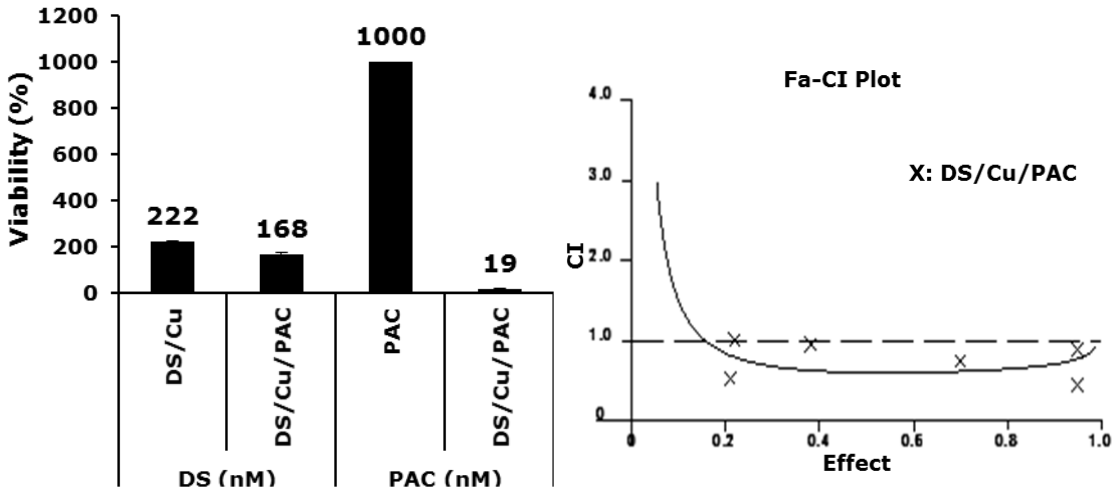


Fig. 4.18 Disulfiram/copper synergistically enhances cytotoxicity of PAC in MDA-MB-231$_{PAC10}$ cells. The cytotoxicity of DS/Cu and PAC in MDA-MB-231$_{PAC10}$ cells is synergistic. The figures represent the mean from three independent experiments. Fa-CI plot of Isobologram analysis. The CI value below 1 indicates synergistic effect between DS/Cu and PAC. The cells were exposed to DS (1μM) and $CuCl_2$ (1μM) for 4 hours and then released in drug-free medium for 24 hours.

4.4 Discussion

In comparison with other BC subtypes, TNBC have poorer chemotherapeutic outcomes with only 12 months of median survival time in advanced TNBC. Although in recent years taxane- and platin-based primary chemotherapy demonstrates high efficacy in TNBC treatment (Frasci *et al*., 2009), drug resistance remains the major barrier for the clinical success of this regimen. Initial treatment of TNBC often leaves residual disease, after that, regrowth TNBC becomes resistant to all

available chemotherapy. This is mainly because the relapsed TNBC has a capacity of pan-resistance, which is similar to the characteristics of drug resistant bacteria (Falagas *et al*., 2008). Pan-resistance is the most disturbing and intractable form of resistance for cancer patients due to the chemoresistance of cancer cells to the whole range of anticancer drugs as well as ionizing radiation. It is very possible that the cancer cells have lost all targetable defects after the initial treatments to protect themselves avoiding cell death or transfer to distant place to regrowth tumour. Three explanations, e.g. mimicry, defence and compensation, of pan-resistance have been proposed by Piet, (Piet *et al*., 2012). Cancer cells can mimic normal cells with some proliferation strategies. Thus cancer cells pretend to be normal cells by imitating rapid growth of host tissues with high turnover rates, making the tumour equally sensitive/resistant to drugs as normal tissues. I would rather prefer the defence and compensation are the case of the pan-resistance rather than the mimicry due to lack of strong evidence to support. Cancer cell defence is the ability to keep all drugs away from their targets. Pgp up-regulation could be part of this defence system by preventing the drug from reaching its target. The mechanism of compensation in the cancer cells is involved in many aspects. Compensation represents adaptations that affect multiple targets, such as increasing the activation of anti-apoptotic pathway, slowing down the cell cycle to avoid drug targeting, and increasing DNA repair. Moreover, CSCs

have been recognised as having an important role in pan-resistance as well as for the cancer relapse. Overall, based on the finding in my study, I will in turn to discuss the characteristic of acquired PAC resistant TNBC cell line (MDA-MB-231_{PAC10}) and how DS target TNBC and reverse the PAC resistant in this cell line.

In comparison with the parental line, the MDA-MB-231_{PAC10} cell line is highly resistant to PAC-induced cytotoxicity (>115-fold), inhibition of clonogenicity (~400-fold) and apoptosis (~75-fold). The MDA-MB-231_{PAC10} cell line is also significantly cross-resistant to CDDP (~2.5-fold, p=0.01). In line with previous reports, Pgp is dramatically overexpressed in the resistant cell line. Although high expression of Pgp plays a role in PAC resistance, MDR1 has no influence on cancer cell sensitivity to CDDP. These results indicate that as a key factor in cell defence mechanism, Pgp may only play a very small part in pan-resistance characteristics in MDA-MB-231_{PAC10} cells. The intrinsic apoptotic pathway plays a main role in both PAC and CDDP induced apoptosis. As a feedback to the chemotherapy drugs induced apoptosis, high background expression of BCL2 protein was detected in MDA-MB-231_{PAC10} cells leading to a higher BCL2/BAX ratio which may desensitize the resistant cell line to PAC and CDDP induced apoptosis.

Furthermore, the resistant cell line proliferates slower with significantly longer doubling time. Flow cytometry analysis demonstrates that a significantly higher population of the resistant cells are blocked in G0/G1 phase with fewer cells entering S-($p<0.01$) and G2/M-phase. PAC exposure induces G2/M-phase arrest in the parental cell line but has no effect on the resistant cell line. It has been known for long time that classical anticancer agents primarily target cycling cancer cells. The quiescent cancer cell population located in G0/G1-phase is resistant to most chemotherapeutic agents and ionizing irradiation. The quiescence has been considered as the main reason for the pan-resistance at the beginning, but it is not an explanation for it because however slow the tumour grows, it will continue to proliferate (Stewart *et al*., 2007; Sijbers *et al*., 1996; Guo *et al*., 2008). PAC is predominately an M-phase-specific drug which stabilizes microtubules causing an M-phase arrest followed by apoptosis (Schiff et al., 1979). Therefore PAC will lose its anticancer activity if the cancer cells are prevented from entering G2/M phase by G0/G1 phase arrest. $p21^{Waf1}$ is a CDK inhibitor inactivating the activity of cyclin A, cyclin E and CDK2 which are essential for G1/S transition. $p21^{Waf1}$ overexpression induces anticancer drug resistance (Bunz *et al*., 1990; Lazzarini *et al*., 2008). Western blot shows that $p21^{Waf1}$ protein is massively up-regulated in the resistant cell line. Because MDA-MB-231 is p53 mutant, the up-regulation of $p21^{Waf1}$ in the resistant cell line is p53 independent. The High $p21^{Waf1}$

expression may be responsible for the G0/G1 block in the resistant cell line. It has been demonstrated that bryostatin-1 induced PAC resistance via up-regulation of $p21^{Waf1}$ (Koutcher *et al*., 2000). Flavopiridol and bryostatin-1 are CDK inhibitors, which slow down cell cycle. After pre-exposure to flavopiridol or bryostatin-1, BC cells become highly resistant to PAC due to flavopiridol and bryostatin-1 induced G0/G1 arrest. The cell cycle disturbance may be, at least partly, responsible for PAC resistance in MDA-MB-231_{PAC10} cell line. Previous studies indicate that overexpression of p21 and cell cycle perturbations can induce CDDP resistance (Koster *et al*., 2010; Wilkins *et al*., 1997). The overexpression of p21 and cell cycle perturbation in MDA-MB-231_{PAC10} cell line may be at least partly responsible for the cross-resistance between PAC and CDDP. These results also support that cell cycle slowing down may contribute to the pan-resistance through its compensation mechanism in cancer cells.

Another concept of pan-resistance has been proposed is CSCs. Cancer cell population contains a small proportion of CSCs. It has been widely accepted that CSCs are responsible for tumour recurrence and display significant resistance to cytotoxic drugs with different acting and resistant mechanisms. It has recently been reported that CSCs are involved in acquired taxane resistance (Domingo *et al*., 2012; McAuliffe *et al*., 2013). In contrast with the fast growing cancer mass, CSCs are quiescent and slow

cycling cells expressing stem cell markers. High expression of Pgp is also a common feature of CSCs. Recent reports indicate that $p21^{Waf1}$ is indispensable for maintaining the quiescent status, stemness and preventing excess DNA-damage accumulation in CSCs. Our findings in MDA-MB-231_{PAC10} cell line (for instance, high p21 expression, cell cycle slowing down, and high expression of Pgp) indicate that this cell line may contain high population of CSCs which may play a key role in the PAC resistance and CDDP cross-resistance. Based upon this hypothesis we examined several other CSC phenotypes. High levels of ALDH, a functional CSC marker, were detected in the resistant cells by ALDEFLOUR analysis. Recent publications (Landen *et al*., 2010; Schafer *et al*., 2012) indicate that high ALDH activity confers chemoresistance upon cancer cells, which can be reversed by targeting ALDH. High expression of the embryonic stem cell-associated genes Sox2, Oct4 and Nanog was also detected in the resistant cell line. Hypoxia induced HIFs overexpression and NFκB pathway activation is responsible for chemoresistance (Wang *et al*., 2004) and also the determinant factors for maintaining stemness of CSCs. Even cultured in normoxic condition, the overexpression and nuclear translocation of HIF2α and NFκBp65 were detected in the resistant cell line. Further studies are being performed in our lab to elucidate the relationship between these factors and CSC-related chemoresistance.

DS is a very efficacious CSC-targeting agent and demonstrates strong chemoresistance-reversing activity. Previous clinical studies manifest that DS and its derivative effectively improve survival of breast and other cancer patients. In this study I examined the direct cytotoxic and PAC-resistance-reversing effect of DS on MDA-MB-231$_{PAC10}$ cell line. Our results show that although MDA-MB-231$_{PAC10}$ cells are resistant to both PAC and CDDP, the cytotoxicity of DS is very comparable in both resistant and sensitive cell lines. DS completely eradicates the clonogenicity in both parental and resistant cell lines. The ALDH activity and the expression of Sox2 and Nanog in the resistant cell line are markedly inhibited by DS exposure. Therefore DS may sensitize MDA-MB-231$_{PAC10}$ cell line to PAC and CDDP by targeting CSCs. The simultaneous inhibition and induction of BCL2 and BAX indicates that DS may induce apoptosis in the resistant cells via an intrinsic pathway. Although DS inhibits MDR1 activity, it has no effect on the expression of Pgp. There is no effect of DS on cell cycle status in the resistant cell line. Similar to many other DNA targeting agents, DS exposure further induces p21 expression in the resistant cells.

In conclusion, in this part of my study, I characterized a newly developed PAC-resistant BC cell line, MDA-MB-231$_{PAC10}$ and investigated the potential PAC resistant mechanisms. Our data show that the resistant cell

line is slow growing and contains a high population of cells with CSC characteristics. I also demonstrated that DS efficiently reverses the PAC and CDDP resistance in MDA-MB-231$_{PAC10}$ cell line. Further study on DS may lead to the discovery of a new efficient chemotherapy agent not only for BC patients but also for all cancer patients.

5. Tackling hypoxia-induced NFκB activation to target breast cancer stem cells

5.1 Introduction

Although mortality has been declining and the median survival rate of BC has been significantly improved in the majority of developed countries due to systemic therapy in early BC treatment, the therapeutic outcomes of A/MBC remain poor with a median 5-year survival rate from diagnosis of only about 23% (Guarneri *et al*., 2004). Chemotherapy is the main arm of treatment for the A/MBC after surgery and radiotherapy. Unfortunately, response duration remains disappointingly short and long-term survival rate remains low. The main limitation for the success of A/MBC treatment is chemoresistance. The relapsed A/MBC is commonly pan-resistant to a

wide range of anticancer drugs, which cannot be explained by individual genetic and biochemical resistant pathways. Therefore tackling the pan-resistance will substantially improve the therapeutic outcomes of A/MBC patients.

CSCs have been considered to have similar characteristics with normal stem cells from tumour-prone tissue. Apparent differences also exist between CSCs and normal stem cells. Normal stem cells are maintained under tight homeostatic regulation and are passively protected in the stem cell niche in adult tissue, whereas CSCs may actively contribute to tumourigenesis through the CSC niche. BC is a highly heterogeneous disease containing a very small fraction (1%) of BCSCs population with stem cell characters (Tirino *et al*., 2013). In 2003, the BCSCs were first discovered when a $CD24^{low}/CD44^{high}$ subpopulation of tumour cells from human patient samples were shown to have tumour-initiating capacity in immunodeficient mice (Al-Hajj *et al*., 2003). Then the development of biomarkers to identify BCSCs as well as the validation of *in vitro* and mouse models has facilitated the isolation and characterization of these cells from murine and human tumours. BCSCs can be identified by detection of stem cell markers (ALDHs, $CD24^{low}/CD44^{high}$) and activation of embryonic related pathways (Sox2, Oct4, Nanog), which are commonly shared with other kinds of cancer (Marques *et al*., 2010). CD44, single-pass

type I transmembrane protein, has been identified as a cellular adhesion molecule for hyaluronic acid, a major component of the extracellular matrix. There are numbers of isoforms that are generated through alternative splicing of CD44 precursor mRNA. The standard isoform of CD44 is expressed predominantly in hematopoietic cells and normal epithelial cell subsets (Aruffo *et al*., 1990; Nagano *et al*., 2004; Ponta *et al*., 2003). Although the exact function of CD44 expression in CSCs remains unclear, CD44 is widely accepted to be associated with EMT and the features of CSCs, e.g. interaction with the corresponding niche (Jin *et al*., 2006), the potential for cell migration and homing (Sackstein *et al*., 2008), the capacity for defense against ROS, and resistance to apoptosis (Shi *et al*., 2012; Toole *et al*., 2008). It has been reported that CD44 expression influences the stem-like properties of CSC populations isolated from the human breast through RNA interference-mediated depletion of CD44 suggesting that CD44 is a potential target for CSC-directed therapy (Pham *et al*., 2011). Furthermore, expression of stemness genes is also used to characterize CSCs. Nanog is a homeodomain-containing transcription factor and, along with the POU domain-containing Oct4 and high mobility group domain-containing SRY Sox2, is part of the key set of transcription factors that have emerged as the master regulatory mechanisms of stem cell pluripotency and differentiation. They are essential for the maintenance of pluripotent embryonic stem cells (Chambers *et al*., 2004; Mitsui *et al*.,

2003; Kashyap *et al*., 2009; Pan *et al*., 2007). Expression of these genes could be a function of malignant transformation and is not unique to BCSC population. Accumulating data show that high expression of these genes could be detected in BC, lung, ovarian cancers and glioblastoma (Tirino *et al*., 2011; Eramo *et al*., 2010).

Human Nanog protein consists of 305 amino acids. Overexpression of Nanog is thought to be an indicator of a poor prognosis for BC patients. In comparison of the non-stemness cancer cells, high expression level of Nanog has been shown in CSCs in BC (Nagata *et al*., 2012). Several studies demonstrated that cancer cells with high levels of the CSC surface marker would possess higher levels of Nanog as well (Xu *et al*., 2012; He *et al*., 2012; Leung *et al*., 2010). It has been considered that Nanog is not only a CSC marker, but also promotes CSC-like characteristics in BC. For instance, overexpression of Nanog increased drug-resistance capacity in BC cell lines and the Nanog protein can bind to the promoter region of cyclin D1 and regulate cell cycle and proliferation (Jeter *et al*., 2011; Choi *et al*., 2012).

Oct4 is a member of the POU family of transcription factors and plays a pivotal role in the maintenance of self-renewal and pluripotency in embryonic stem cells. Oct4 is expressed in many malignant tumours and the expression profile has been related to tumour grade and disease

progression (Huang *et al*., 2012; Zhang *et al*., 2010c). It has been reported that Oct4 expression is present in both normal adult stem cell and CSCs (Tai *et al*., 2005). An important study has demonstrated that high expression of Oct4 in normal breast cells led to the generation of cells with tumour-initiating and colonization abilities. These cells developed high-grade, poorly differentiated BC in nude mice (Beltran *et al*., 2011). Furthermore, high Oct4 expression has been shown to enhance the features of CSCs in a mouse model of BC (Kim *et al*., 2011).

Sox2 is a member of a superfamily of proteins that all possess a high mobility group (HMG) box DNA-binding domain and it is pivotal for early development and maintenance of undifferentiated embryonic stem cells. A recent study showed that two cell subsets have been identified based on differential Sox2 transcription activity in MCF7 and ZR75 BC cell lines, and subset possessing Sox2 transcription could significantly form more colonies in methylcellulose and more mammospheres *in vitro* whereas knockdown Sox2 in the subset abolished these abilities (Wu *et al*., 2012). Therefore, it can be concluded that Sox2 transcription activity was responsible for the tumourigenicity and CSCs phenotypes in BC (Wu *et al*., 2012). Moreover, down-regulating Sox2 drastically reduced their transformed properties *in vitro* and BC cells with depletion of Sox2 couldn’t form mammospheres and differentiation any more. All these

studies indicated that Sox2 expression promoted and maintained the stemness of CSCs.

CSCs are responsible for chemoresistance and tumour relapse. CSCs are relatively quiescent, express multidrug resistant and anti-apoptotic proteins (Storci *et al*., 2010; Simsek *et al*., 2010). Investigation of CSC character will contribute to understanding the role of CSC in therapeutic failure. Further study on CSC may develop a novel specific targeting drug for CSC to prolong the survival rate for cancer patient. It is widely understood that the CSC is more than a functional unit. Emerging evidence suggests that CSC resides in a hypoxic/necrotic tumorous area named as CSC niche, which controls self-renewal and differentiation of CSCs. Moreover, CSCs can be generated in the niche for maintenance purposes through induction of CSC features. The niche is also recognized involved in metastasis by induction of the EMT, leading to dissemination and invasion of tumour cells. The secondary tumours also seem to initiate in the premetastatic niche. Therefore, the microenvironment plays a crucial role in primary tumour growth as well as metastasis formation (Bao *et al*., 2012). Low oxygen in the niche of the cancer cells termed hypoxia, which is one of the most pervasive microenvironmental stresses and has been recognized as the most common features of solid tumours. Clinically, hypoxia and its signalling pathway have been shown to be associated with radio- and

chemo-resistance, contributing to increased risk of tumour recurrence and metastasis, leading to reduced overall survival rate and increased mortality (Jubb *et al*., 2010). A wide range of studies has also demonstrated that hypoxia plays a pivotal role in the maintenance of the stemness of CSCs (Thomlinson *et al*., 1955; Brown *et al*., 1998). The hypoxia-induced and retained CSC characteristics can be reversed into differentiated phenotype in normoxic condition and vise versa. *In vitro*, the CSC and non-CSC phenotypes are interconvertible in spheroid and monolayer culture respectively (Zeng *et al*., 2011). Therefore the stemness of CSCs is highly environment-dependent and the environmental oxygen concentration plays determinant roles in the maintenance of the stemness in CSCs (Cummins *et al*., 2005). Although most publications emphasize the importance of the HIFs in induction and maintenance of CSC phenotypes, the detail molecular mechanistic relationship between hypoxia and CSCs is still not fully elucidated. Approximate 20 different transcription factors are induced directly or indirectly by hypoxia including NFκB (Arlt *et al*., 2003). NFκB is a transcription factor, which plays an important role in cell survival, proliferation, invasion, migration, angiogenesis, tumour metastasis and chemoresistance (Wang *et al*., 2007; Walmsley *et al*., 2005). Moreover, emerging evidence suggests that NFκB also plays an important role in EMT and CSCs, contributing to tumour aggressiveness (Sarkar *et al*., 2008;

Widera *et al*., 2008). Hypoxia could activate NFκB signalling pathway in a variety of cells including cancer cells. In most normal cells, NFκB is sequestered in the cytoplasm as an inactive complex through the direct binding to its natural inhibitor, the inhibitor of NFκB (IκB). Upon various stimuli, IκB will be phosphorylated by IκB kinase complex (IKKα,β,γ), ubiqutinated and promptly degraded which releases NFκB from NFκB-IκB complex. The liberated NFκB dimers are then translocated into the nucleus, binding to the promoter region of the relevant downstream genes and triggering a series of molecular reactions (Scortegagna *et al*., 2008).

It is believed that high activation of NFκB inhibited apoptosis to enable a hypoxic cell to survive through the period of hypoxic insult. The hypoxic action of NFκB signalling pathway also involves promoting hypoxic inflammatory response through the regulation of gene expression of proinflammatory cytokines as well as adhesion molecules, enzymes, and pro-inflammatory enzymes (Taylor *et al*., 2008; Taylor *et al*., 2009). Furthermore, it is reasonable to speculate that hypoxia induced NFκB activation may be contributing to the maintenance of CSCs during the development and progression of tumours especially because both HIFs and NFκB signalling pathways are known to maintenance of stem cell phenotype and function in tumour niche. Hypoxic activation of NFκB pathway may be HIF1α-dependent. The functional loss of HIF1α could

decrease NFκB activation and p65 expression whereas increased expression of HIF1α results in the activation of NFκB through hyper phosphorylation of IκB and phosphorylation of p65 at Ser^{276} in keratinocytes (Scortegagna *et al*., 2008). The molecular mechanism of how the HIF pathway regulates the NFκB pathway has not been fully understood, but HIF pathway activates NFκB pathway mainly through the regulation of IKK signalling-mediated regulation of canonical NFκB signalling (Cummins *et al*., 2006) In addition to HIF-mediated activation of NFκB, it has recently been demonstrated that hypoxia can directly induce NFκB which in turn regulates HIF pathway. The promoter region of HIF1α contains κB site. The mutation of this κB site will lead to the loss of hypoxia-induced HIF1α activation (Van *et al*., 2008). Moreover, blocking individual NFκB members by their specific siRNAs can decrease HIF1α activity. The activation of NFκB induced by TNFα and p50/p65 transfection lead to increased levels of HIF1α mRNA and protein. Therefore NFκB can regulate HIF1α signalling pathway to maintain the basal levels of HIF1α under normoxic condition and further induce it under hypoxia (Van *et al*., 2008; Bracken *et al*., 2005). An earlier report demonstrated that NFκB also induces HIF2α activation via influence of the interaction between IKKγ and CBP/p300 (Tian *et al*., 2012; Schreck *et al*., 1992). It has been reported that the interaction of HIF and NFκB pathways

contributes to BC metastasis through the induction of EMT together with migration through p65-lysine acetylation and HDAC dependent epigenetic mechanism to up-regulate NFκB and HIF (Bendinelli *et al*., 2009). In summary, the interaction between HIF and NFκB signalling pathways plays a pivotal role in tumour aggressiveness.

Recent studies indicate that NFκB plays a pivotal role in hypoxia-induced CSC phenotypes and is responsible for the chemoresistance in CSCs. Although the CSC concept is still debatable (Yip *et al*., 2011), it is widely accepted that the cancer cells expressing CSC markers are resistant to chemo- and radiotherapy. Therefore, development of anti-CSC drugs to target CSC determinant pathways will improve chemotherapeutic outcomes. DS is a commercially available anti-alcoholism drug (Chen *et al*., 2006) and shows anticancer activity *in vitro* and *in vivo* (Wang *et al*., 2003; Guo *et al*., 2010). It also potentiates cyclophosphamide, CDDP and radiation *in vitro* and protects normal cells in kidney, gut and bone marrow *in vivo* whilst increasing the therapeutic index of cytotoxic drugs (Liu *et al*., 2012; Dufour *et al*., 1993). The anticancer activity of DS is Cu dependent. Cu plays a crucial role in redox reactions and triggers the generation of ROS in human cells. DS/Cu is a strong ROS inducer and proteasome-NFκB pathway inhibitor. Combination of DS with Cu may target cancer cells by simultaneous modulation of both ROS and NFκB. DS and its metabolites

can also covalently modify cysteine residues within the nucleotide binding domain of Pgp and permanently inhibit Pgp activity (Hinohara *et al*., 2012). This will potentially reverse multidrug resistance. Although the anticancer activity of DS has been reported for a long time, there are very few successful cases reported in clinic. This discrepancy is mainly introduced by the very short half-life of DS in the bloodstream. The currently developed technologies in nano-encapsulation may be able to translate this drug into cancer indication.

In this study, I examined the effect of hypoxia on maintenance of stemness of CSCs and elucidated the bridging role of NFκB in linking hypoxia and CSCs. Furthermore, the anticancer efficacy of a newly developed liposome encapsulated DS have also examined in BC cell lines and BC xenografted nude mice.

5.2 Methods

5.2.1 *In vitro* mammosphere and suspension culture and cytotoxicity assay

The BC cells were cultured in ultra-low adherence 6-well plates (Corning, MA, USA) containing 2 ml of stem cell culture medium [SCM, serum-free DMEM-F12 supplemented with B27 (Invitrogen, Paisley, UK), 20 ng/ml

epidermal growth factor (Sigma), 10 ng/ml basic fibroblasts growth factor (R & D System, Abingdon, UK), 10 μg/ml insulin (Sigma)] at a density of 10,000 cells/ml. For the suspension culture, the cells were cultured in full medium (DMEM supplemented with 10% FCS, 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin). After 6 days culture, the mammospheres and suspending cell balls were photographed and subjected to further treatments. For in vitro cytotoxicity assay, the cells cultured in suspension or mammospheres were trypsinised and seeded in 96-well plates at a density of 5,000 cells/well and exposed to drugs for 72 hours before MTT assay.

5.2.2 Measurement of hypoxia in cell culture

The hypoxic status was determined using the Hypoxyprobe^{TM-1} Plus Kit supplied by Hypoxyprobe Inc (Burlington, MA, USA) following the supplier's instruction. For immunocytochemistry assay, the cells were cultured in 8-well chamber slides at normoxic (20% oxygen) or hypoxic [1% oxygen in Hypoxic Chamber (StemCell, Durham, NC, USA)] conditions for 24 hours and labelled with Hypoxyprobe for 2 hours. The hypoxic cells were detected by confocal microscope after staining with FITC-conjugated anti-hypoxyprobe Mab. The mammospheres and cells in suspending condition were cultured for 6 days. The mammospheres and suspended cultured cells were labelled with Hypoxyprobe for 24 hours and

cytospinned at 800 g for 3 minutes to spread the spheres onto Polylysine-coated slides (VWR, Lutterworth, UK). For flow cytometric analysis, the cells were cultured in 25 cm flasks at the above conditions. After immunocytochemistry staining the cells were subjected to flow cytometric analysis. The hypoxic population was detected using a FACSCalibur flow cytometer with 488 nm blue laser and standard FITC 530/30 nm bandpass filter. To determine the effect of hypoxia on drug sensitivity, the cells were cultured in 1% oxygen condition at a cell density of 1 x 10^3 cells/well in 96-well plate for 4 days and exposed to anticancer drugs for another 72 hours before MTT assay. The parallel MTT assay was performed in normoxic condition.

5.2.3 Immunofluorescent flow cytometric analysis of embryonic stem cell markers

The expression of Nanog, Oct4 and Sox2 was determined by immunofluoresent flow cytometry. The sphere and hypoxia-cultured cells were collected by trypsinization. The cells fixed by acetone/methanol and permeabilized by 0.1% triton-X100. After being blocked with 3% BSA for 1 hour the cells were stained with primary (1:50 dilution) and FITC-conjugated secondary antibodies respectively for 1 hour at RT. The positively stained population was detected using a FACSCalibur flow

cytometer with 488 nm blue laser and standard FITC 530/30 nm bandpass filter.

5.2.4 Total RNA isolation and RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Paisley, UK) according to the manufacturer's protocols. The mRNA expression levels of ALDH 1A1 (NM_000689), 1A3 (NM_000693), 2 (NM_000690) and 3A1 (NM_000691) genes were determined using the Access RT-PCR System (Promega, Southampton, UK) following the instruction of the supplier. The human housekeeping gene GAPDH (XR018317) was used as the RNA loading control. The sequences of the primers and the sizes of the amplified fragments are table 2.2. The RT-PCR amplification conditions were as follows. One cycle at 48℃ for 45 minutes; 1 cycle at 94℃ for 2 minutes; 35 cycles at 94℃ for 30 seconds, 60℃ for 30 seconds, 72℃ for 1 minute; 1 cycle at 72℃ for 5 minutes. The RT-PCR products were separated on a 1% agarose gel and the bands visualized and photographed under UV light.

5.2.5 Human breast cancer xenograft experiments

Five-week-old female BALB/c Nu/Nu athymic nude mice (Biotechology & Cell Biology Shanghai, China) were housed under pathogen-free conditions according to Fourth Military Medical University (FMMU), China animal care guidelines and the animal experiments were reviewed and approved by

the Ethical Committee of FMMU. MDA-MB-231 cells (1 x 10^6) were subcutaneously injected at one flank of the mice. When the tumour volume (V) reached ~200mm^3, the tumour bearing mice were randomly subdivided into three groups (8 mice/group) and treated 2 times/week with Lipo-DS (75 mg/kg) empty liposome (0.2 ml) and no treatment. The tumour volume was calculated by the following formula: $V= (L \times W^2) \times 0.5$, where L is the length and W is the width of the tumour. The xenograft size was observed twice per week for 4 weeks. After 4 weeks or when the xenograft reached 1500mm^3, the animals were sacrificed. The tumours were removed, photographed and subjected to further analysis.

5.2.6 Immunohistochemistry

The tumour and normal tissues were paraffin embedded. After deparaffinization and rehydration, the slide was blocked its endogenous peroxidase by 3% hydrogen peroxide, incubated with primary antibody Ki67 antibody (1:200, Cell signaling), ALDH1 (1:100, ABCam), NFκBp65 (1:200, Cell Signaling) then with biotinylated secondary antibody, antimouse immunoglobulin G (H + L), followed by incubation in ABC reagent (Avidin and Biotinylated horseradish peroxidase Complex, DAKO Labs, Cambridgeshire, United Kingdom). Finally, the slide was mounted with 3, 3'-diaminobenzidine and visualized under a microscope.

5.2.7 H&E staining

Paraffin-embedded sample slides were deparaffinized and hydrated, and then stained with hematoxylin for 1 minute. After rinsing, the slides were then stained with eosin for 1 minute, followed by more rinsing, and coverslips were mounted onto slides with Permount.

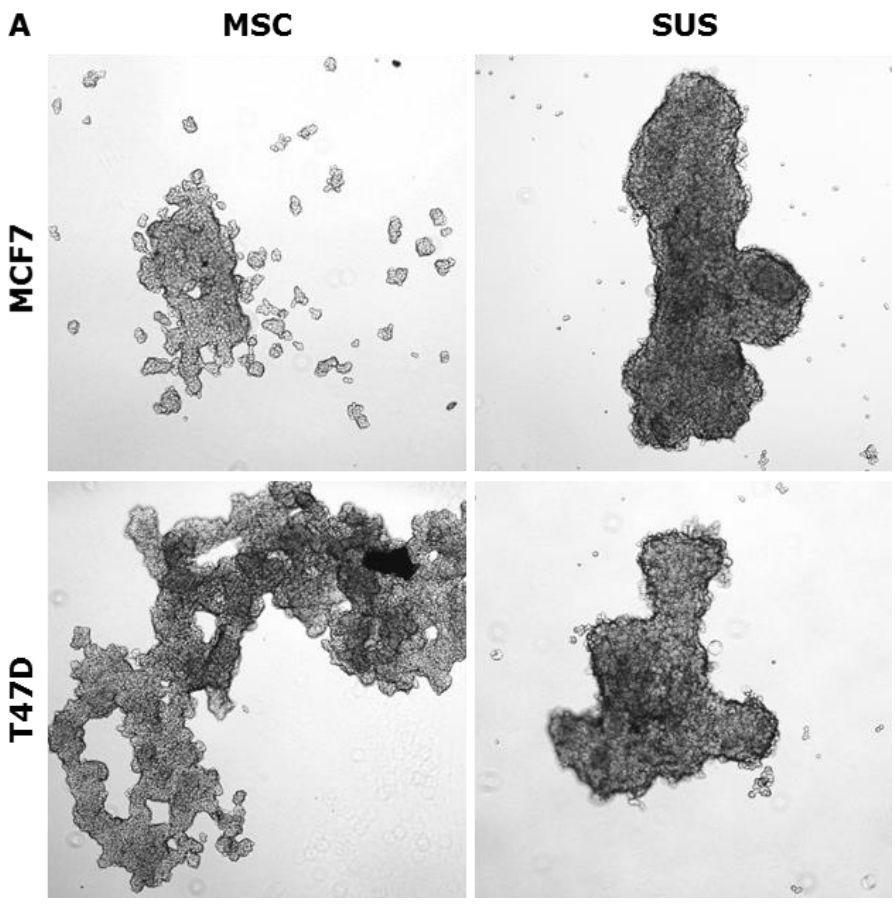
5.2.8 Terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labelling assay

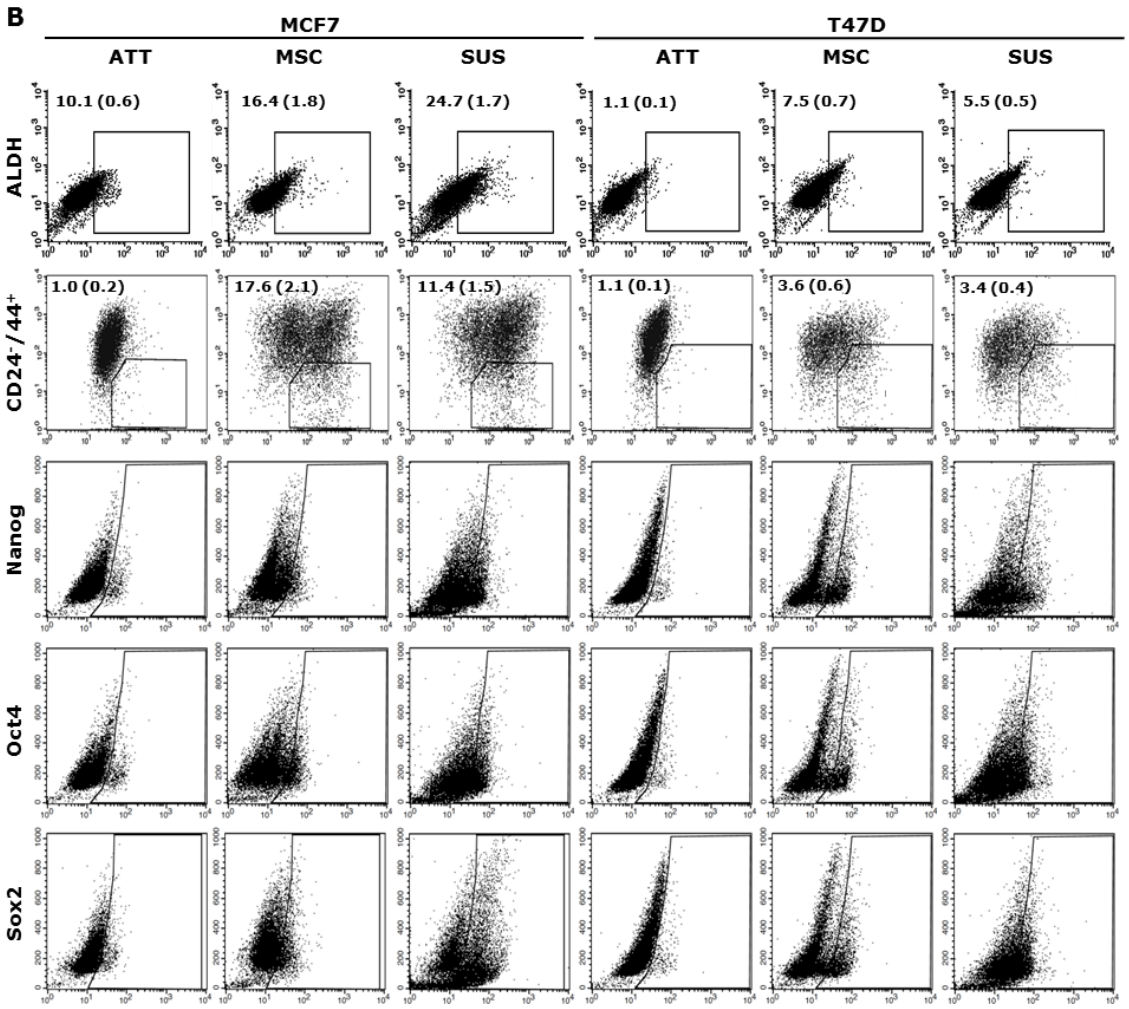
Tumour tissues were paraffin embedded and stained according to the instructions of the manufacturer. Briefly, after deparaffinization and hydration, the tissue was incubated with Working Strength TdT Enzyme, Working Strength Stop/Wash Buffer, conjugated with antidigoxigenenin, and then stained with peroxidase substrate and incubated in ABC reagent (Avidin and Biotinylated horseradish peroxidase Complex, DAKO Labs, Cambridgeshire, UK). Finally, the slide was mounted with 3, 3'-diaminobenzidine and visualized under a microscope.

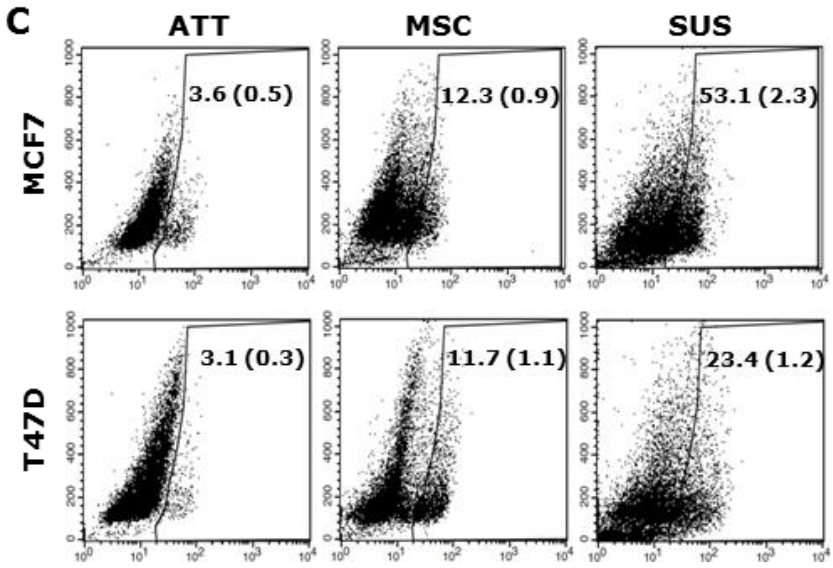
5.3 Results

5.3.1 Hypoxia is responsible for maintaining stemness and drug resistance in cancer stem cells and suspension cells

The *in vitro* MSC culture conditions are adopted from normal stem cell culture system, but serum-free system is very costly and does not very well mimic the physiological condition. In this study, I firstly examined if serum-free is an essential condition to maintain the stemness of MSC *in vitro.* Two BC cell lines were cultured in parallel in both classical serum-free spheroid stem cell culture system (MSC) and normal serum-rich medium (SUS). After 5 days culture, BC cells formed typical mammospheres in both conditions. The spheres formed in the serum-rich medium are markedly tighter than their counterparts cultured in serum-free stem cell medium (Fig. 5.1A). Furthermore, both MSC and SUS cells have significantly higher proportion of cells expressing stem cell markers ($ALDH^+$ and $CD24^{low}/CD44^{high}$) then the attached cells and CSC related embryonic proteins (Sox2, Nanog and Oct4). In comparison with the MSC, most of the stem cell-related markers are expressed at higher levels in the SUS cells (Fig. 5.1B). Fig. 5.1C and D demonstrate that in comparison with the adherent cells, a high population of hypoxic cells were detected in both MSC and SUS cells using HypoxyProbe.


A
MSC
SUS
MCF7
T47D


B
MCF7
T47D
ATT
MSC
SUS
ATT
MSC
SUS
ALDH
10.1 (0.6)
16.4 (1.8)
24.7 (1.7)
1.1 (0.1)
7.5 (0.7)
5.5 (0.5)
CD24-/44+
1.0 (0.2)
17.6 (2.1)
11.4 (1.5)
1.1 (0.1)
3.6 (0.6)
3.4 (0.4)
Nanog
Oct4
Sox2


C
ATT
MSC
SUS
MCF7
3.6 (0.5)
12.3 (0.9)
53.1 (2.3)
T47D
3.1 (0.3)
11.7 (1.1)
23.4 (1.2)

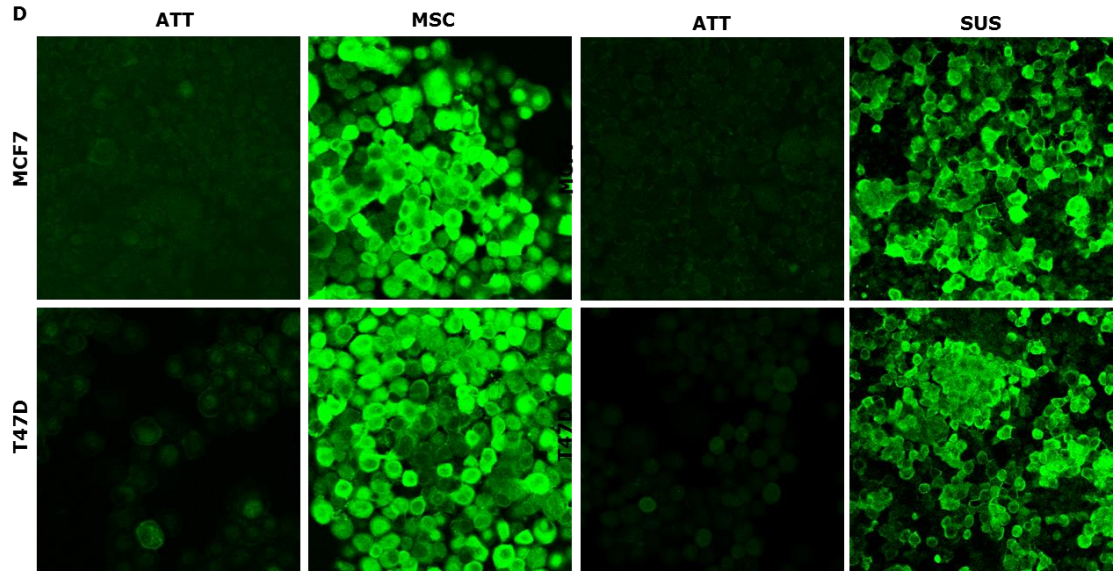
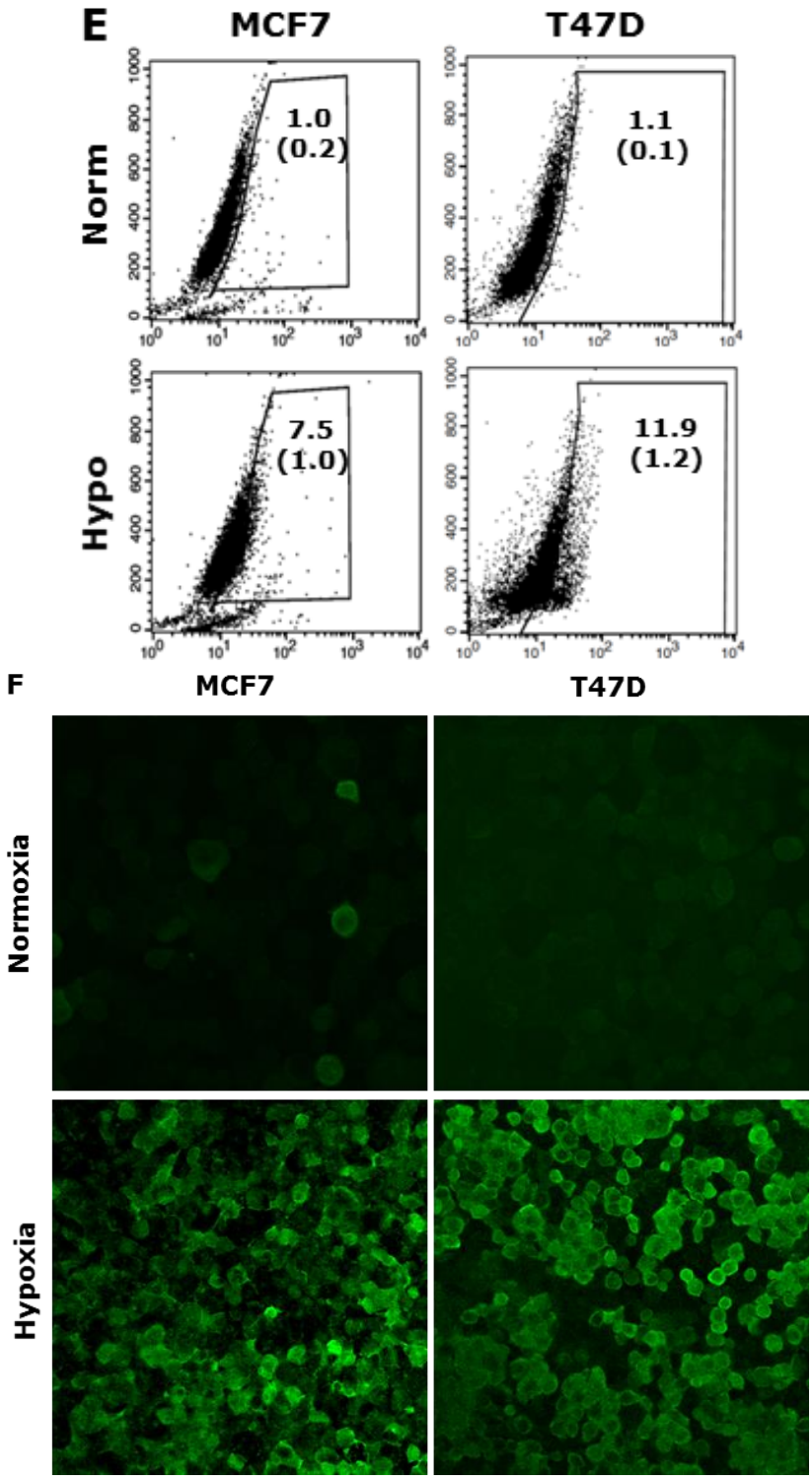


Fig. 5.1 Hypoxia induces expression of stem cell markers in breast cancer cell lines. (A) The morphology of spheres cultured in serum-free (MSC) and serum-containing (SUS) medium (40X magnification). (B) Flow cytometric analysis of ALDH activity and expression of CD24, CD44, Oct4, Sox2 and Nanog proteins in monolayer- and suspension-cultured cells. (C) Flow cytometric analysis of hypoxic cells stained with Hypoxyprobe. (D) Confocal microscopy images of the hypoxic cells detected by Hypoxyprobe in serum-free (MSC) and serum-containing (SUS) medium cultured BC cells (X400 magnification). ATT: monolayer culture; MSC: serum-free stem cell culture; SUS: serum-containing medium culture in suspension. The figures in the frame represent Mean (SD).

It has been reported that the hypoxic condition in the stem cell niche is essential for maintaining the stemness. I hypothesized that the sphere shape growth may prevent penetration of oxygen into the centre of the mammospheres and a relatively hypoxic condition will be generated in the centre of the spheres. Therefore I cultured both cell lines in 1% oxygen condition for 5 days to determine the relationship between hypoxia and CSC characters. The results show that similar to the sphere cells, the

hypoxic condition-cultured monolayer cells express high levels of stem cell markers and embryonic pathway-related proteins (Fig. 5.1E, F and G).



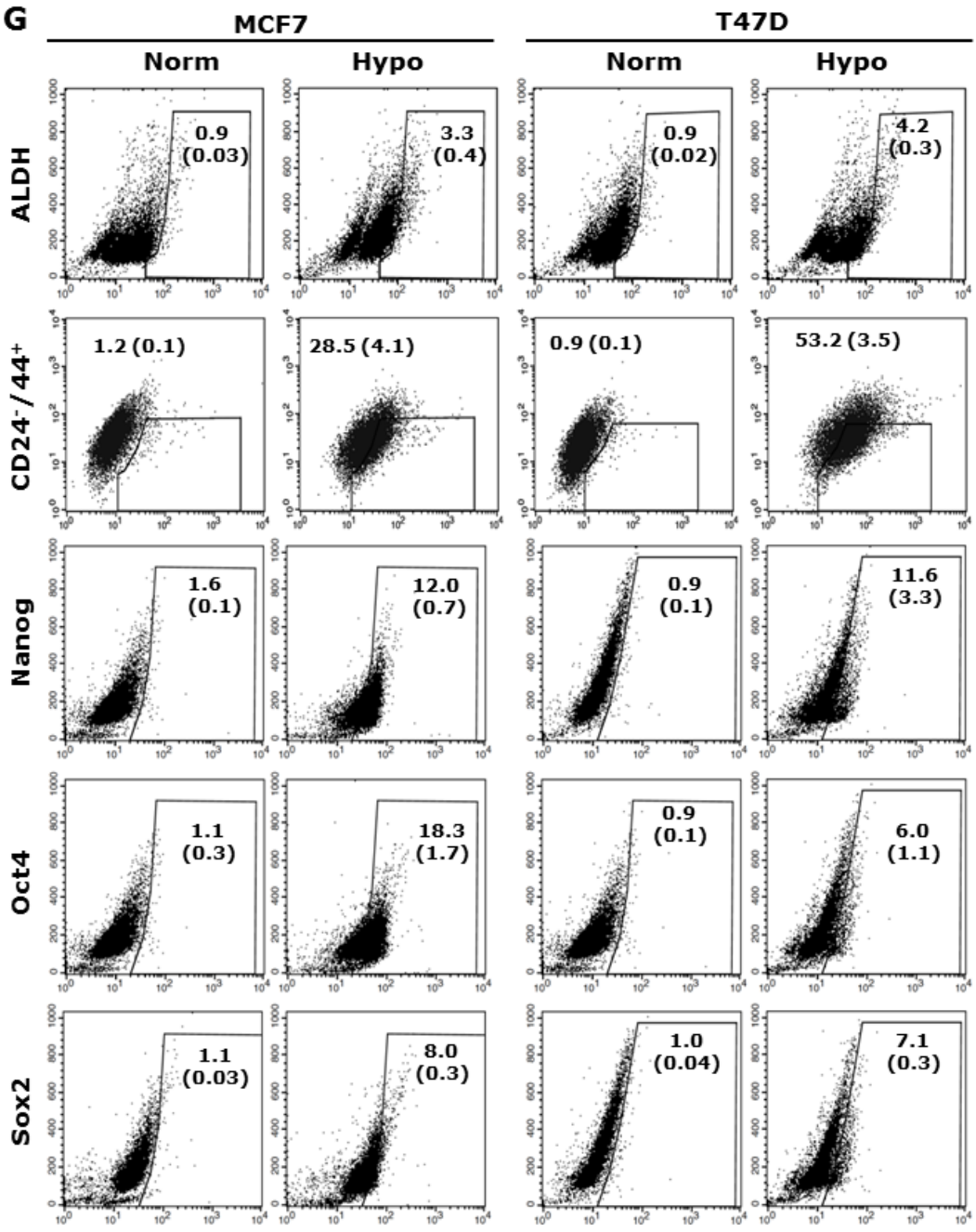


Fig. 5.1 Hypoxia induces expression of stem cell markers in BC cell lines. (E) Hypoxyprobe stained hypoxic population in hypoxia-cultured BC cells was detected by flow cytometry and immunocytochemistry (F) respectively. (G) Flow cytometric Comparison of ALDH activity and expression levels of CD24, CD44, Oct4, Sox2 and Nanog proteins in normoxia- and hypoxia-cultured cells. ATT: monolayer culture; MSC: serum-free stem cell culture; SUS: serum-containing medium culture in suspension. The figures in the frame represent the Mean (SD).

CSCs are commonly *de novo* resistant to a wide range of anticancer drugs and become the source of cancer recurrence. I further examined the sensitivity of the cells cultured in both systems. Table 5.1 shows that BC cells cultured in both systems are highly resistant to three first line anti-BC drugs. The above results suggest that the culture medium has no effect on cancer cell sensitivity to anticancer drugs. Similar to the CSC and SUS cells, the cells cultured in hypoxic condition are significantly resistant to chemotherapeutic agents (Table 5.1). All of these data indicate that hypoxia may play a key role in determining the stemness and chemosentivity in BC cells.

Table 5.1 Cytotoxicity of conventional anticancer drugs in breast cancer cell lines

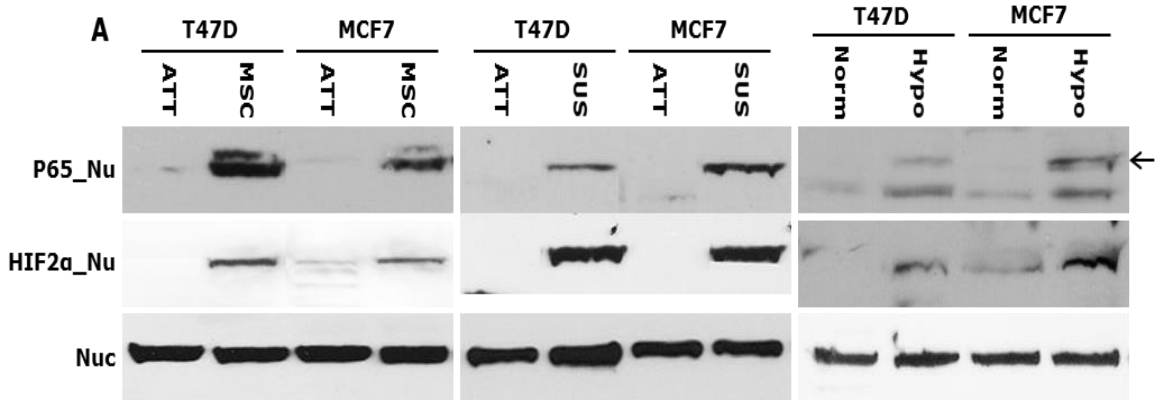
	MCF7			T47D		
	dFdC	Dox	PAC	dFdC	Dox	PAC
ATT	28.7 (7.6)	17.1 (1.1)	6.3 (1.6)	76.3 (5.7)	64.2 (2.4)	7.7 (2.4)
MSC	>1000	>2000	>1000	>1000	>2000	>1000
SUS	>1000	>2000	>1000	>1000	>2000	>1000
Hypo	>1000	>2000	>1000	>1000	>2000	>1000
Mock	14.8 (5.2)	63.2 (12.9)	2.8 (0.4)	2.5 (2.0)	169.8 (42.5)	6.2 (1.8)
C2	>1000	424.3** (65.5)	>1000	N/A	N/A	N/A
P1	>1000	159.8** (36.8)	>1000	N/A	N/A	N/A
C1	N/A	N/A	N/A	>1000	498** (130)	>1000
C3	N/A	N/A	N/A	>1000	>1000	>1000

The figure represents IC50 value from three MTT experiments [mean (SD)]. The cells were exposed to drugs for 72 hours. ATT: attached cells; MSC: mammosphere cells; SUS: suspension culture in normal medium; Hypo: hypoxic culture (O2 < 1%). dFdC: gemcitabine, DOX: doxorubicin, PAC:

paclitaxel. Mock: Empty vector transfected cells; C2, P1, C1, C3: NFκB p65 transfected clones. ** $p<0.01$ (n=3) compared with Mock cells.

5.3.2 NFκB activation plays pivotal role in maintaining cancer stem cell stemness and chemoresistance

In this study, high nuclear expression of HIF2α was detected in the cells cultured in hypoxia, CSC and SUS conditions. In line with this alteration, p65 nuclear translocation has been observed in all of these three culture conditions (Fig. 5.2A). Phosphorylation of NFκB p65 subunit and AKT was also detected in these cells (Fig. 5.2B). EMSA demonstrated that the NFκB DNA binding activity was induced in the cells cultured in these three conditions. These results indicate that both hypoxia and NFκB pathways were activated in all of these three conditions.



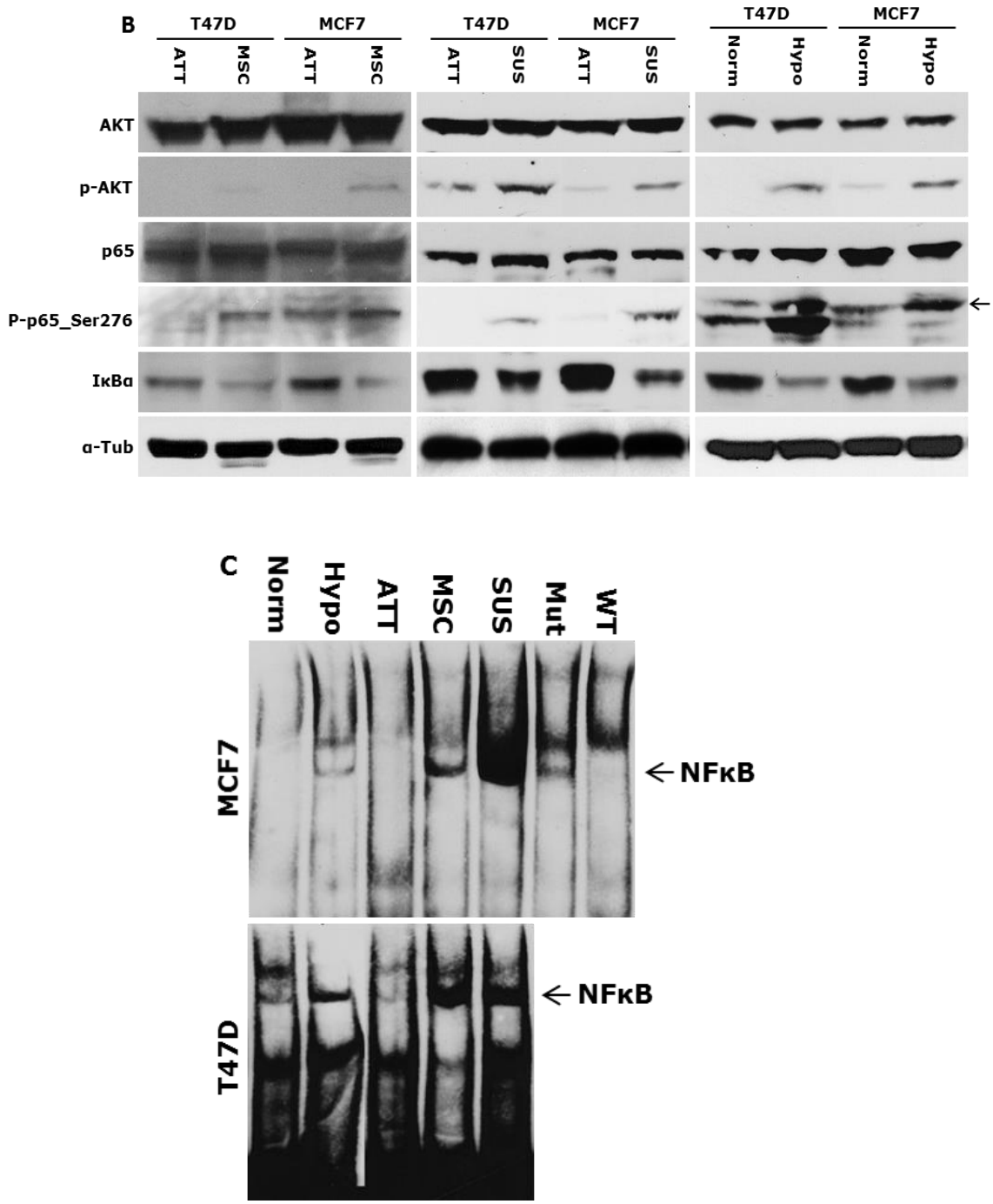
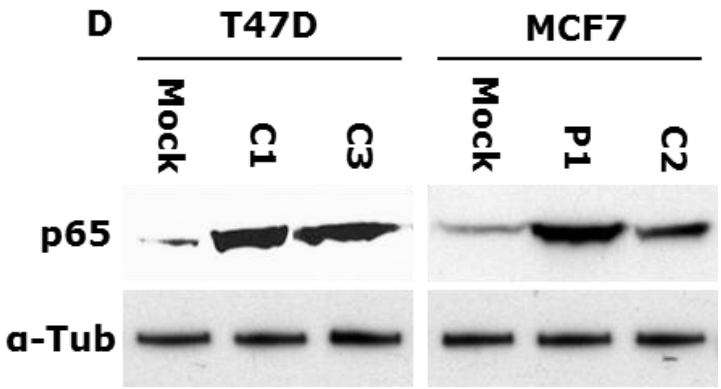
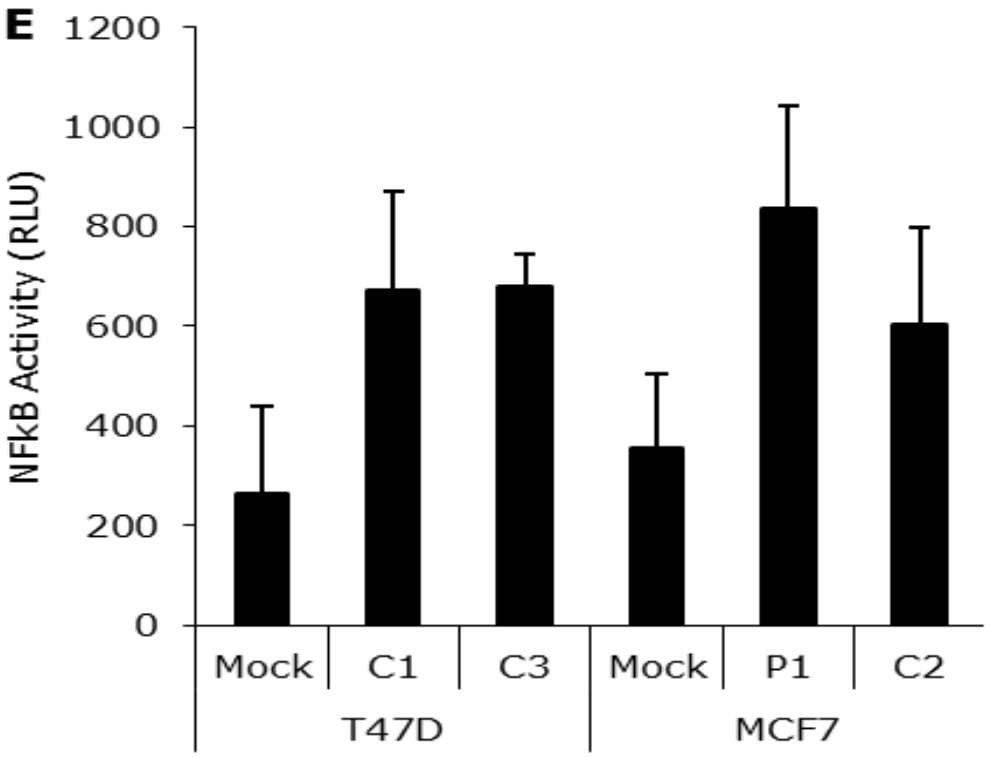


Fig. 5.2 NFκB is responsible for maintaining cancer stem cell characters in suspension-cultured and hypoxic cells. (A) High levels of NFκB p65 and HIF2α protein were detected in the nuclear protein extracted from suspension-cultured and hypoxic cells. Nuc: nucleolin used as a loading standard. (B) Phosphorylated AKT, NFκB p65_Ser276 and degradation of IκBα were detected in suspension-cultured and hypoxic cells by western blot. α-Tub: α-tubulin used as a

loading control. (C) High NFκB DNA binding activity was detected by EMSA. Mut and WT: mutant and wide type probe competition. High NFκB p65 protein

To examine the determinant role of NFκB in maintenance of stem cell characters and chemosensitivity, I transfected both MCF7 and T47D cell lines with the cDNA of NFκB p65 subunit. High p65 protein levels and transcriptional activity of NFκB were detected in the transformed clones (Fig. 5.2 D and E). Flow cytometry data show that all the transformed clones possess considerable higher population of cells with CSC markers (Fig. 5.2F). Furthermore I examined the chemosensitivity in the transfected cell lines (Table 5.1). In comparison with the mock transfected cell lines, the p65 transfected cell lines are highly resistant to three first line anti-BC chemotherapeutic agents (DOX, PAC and dFdC). These data indicate that NFκB may play a key role in the network of hypoxia-induced CSC characters and cancer chemoresistance.




E
1200
1000
800
600
400
200
0
NFkB Activity (RLU)
Mock
C1
C3
Mock
P1
C2
T47D
MCF7

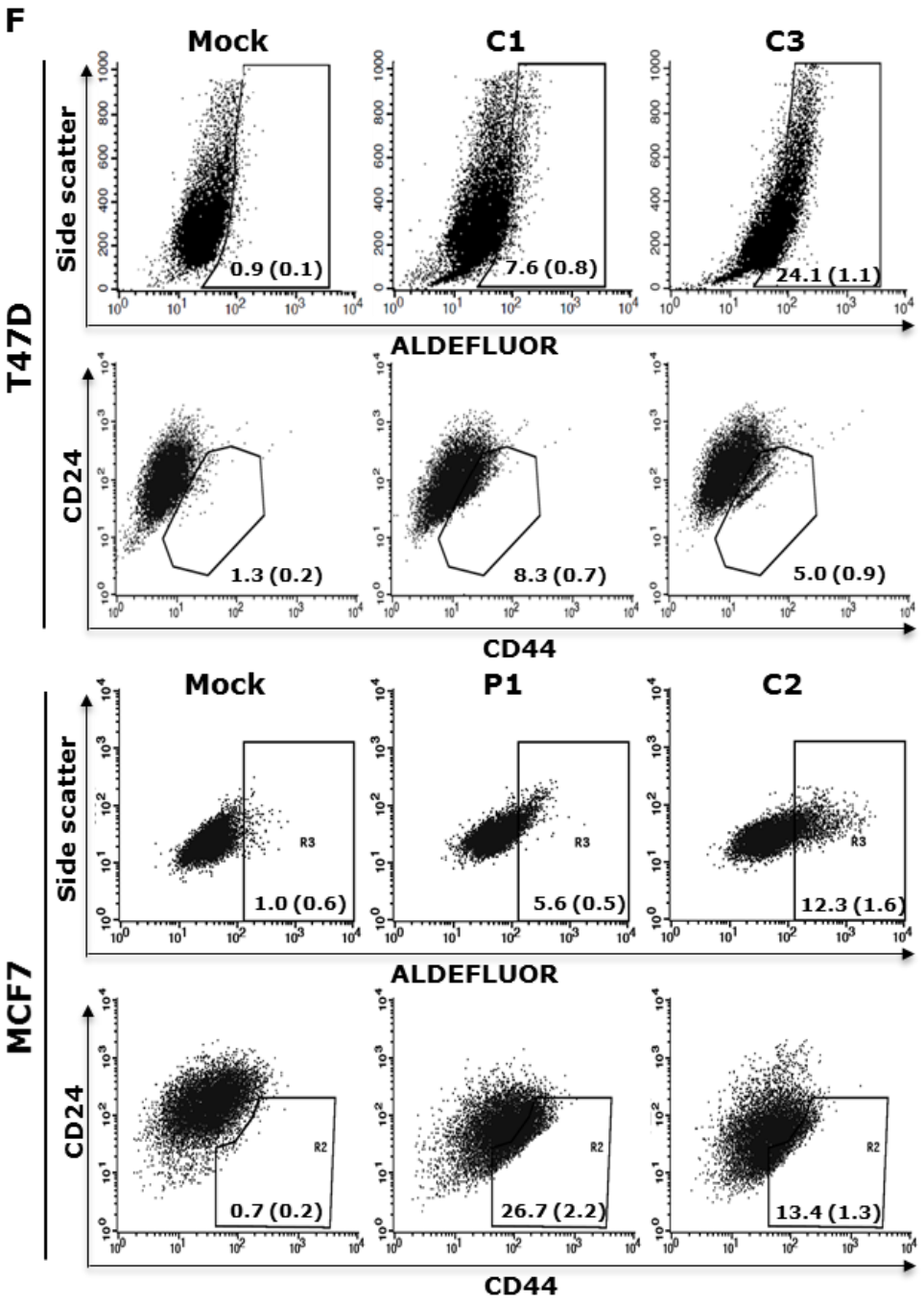
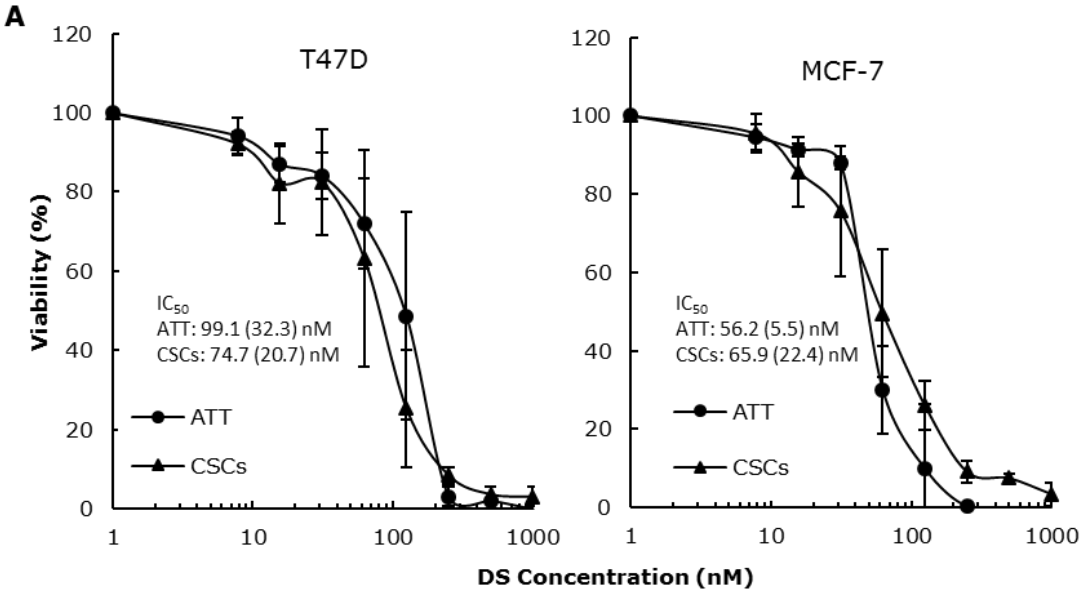
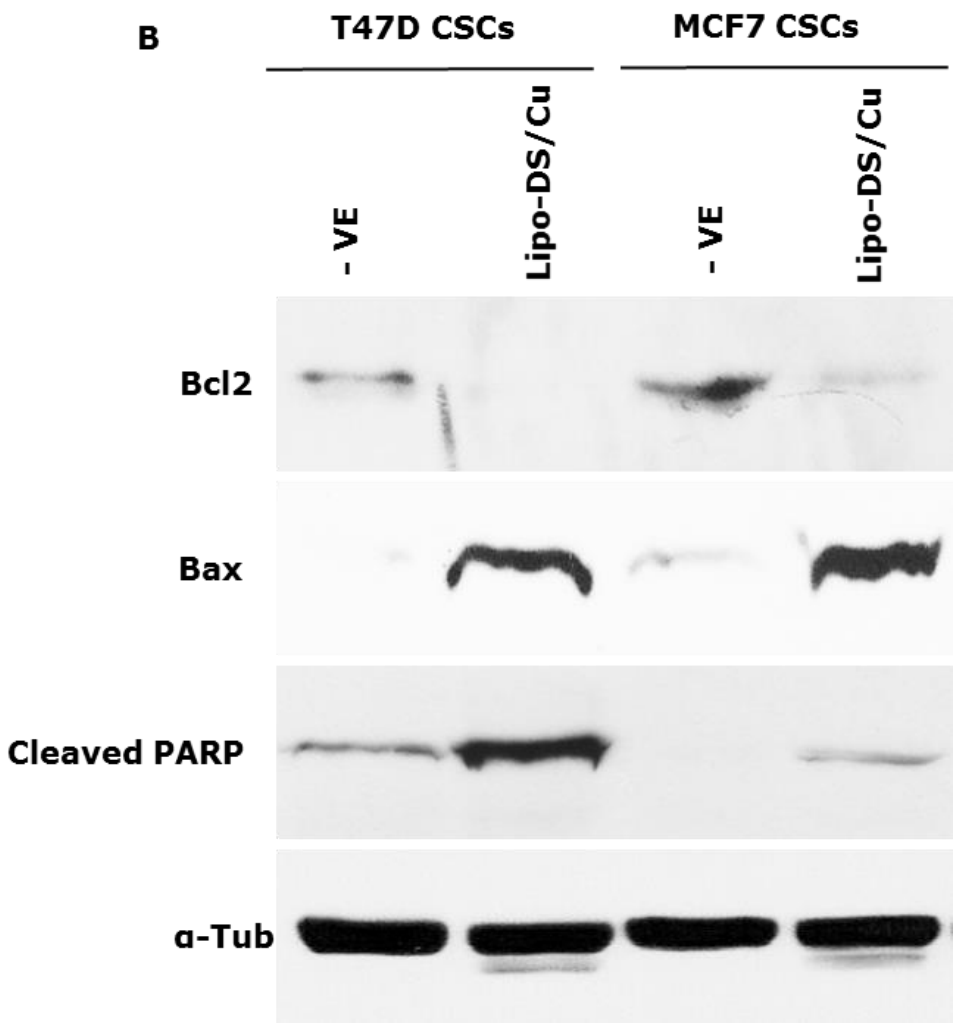


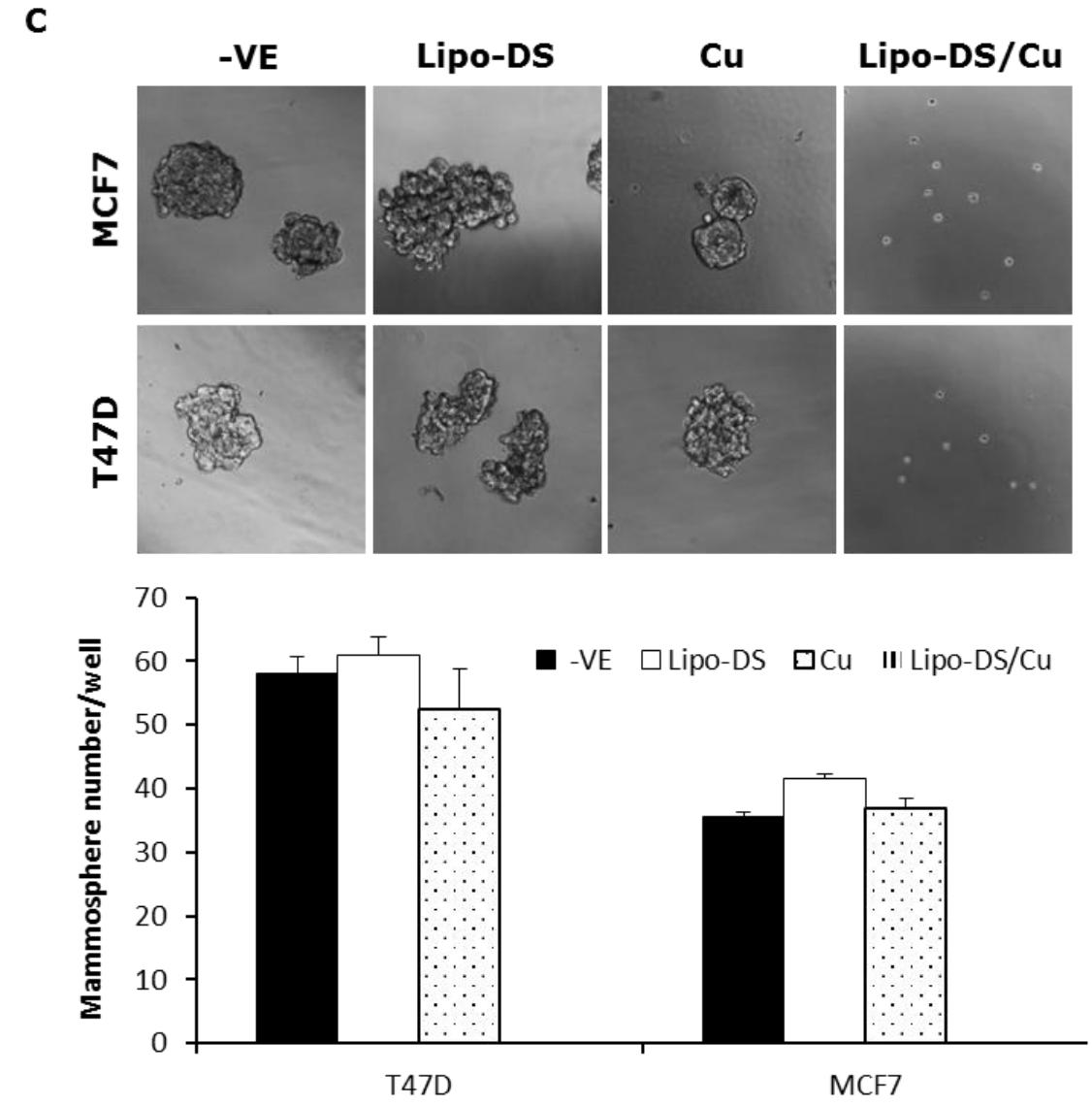
Fig. 5.2 NFκB is responsible for maintaining cancer stem cell characters in suspension-cultured and hypoxic cells. (D) and transcriptional activity (E) were detected in p65 transfected clones (C1, C3, P1 and C2) by western blot and luciferase reporter gene assay respectively. Mock: empty vector transfected cells. (F) High $ALDH^{+}$ and $CD24^{low}/CD44^{high}$ population was detected in NFκB p65 transfected clones. The figures in the frame represent Mean (SD)

5.3.3 Lipo-disulfiram targets cancer stem cells *in vitro*

In line with my previous results, I further examined the toxicity and specificity of Lipo-DS in BC cell lines in combination with 10 μM of $CuCl_2$. Lipo-DS is high cytotoxicity to the CSCs derived from MCF7 and T47D (Fig. 5.3A). Lipo-DS/Cu induced and inhibited the expression of BAX and BCL2 respectively (Fig. 5.3B). The clonogenicity in both MCF7 and T47D cell lines was completely blocked after 1 hour Lipo-DS/Cu treatment. The sphere-forming ability in two BC cell lines was also completely inhibited after 4 hours Lipo-DS/Cu treatment. This effect was not observed in Lipo-DS or Cu treated cells (Fig. 5.3C). Lipo-DS/Cu also abolished the $ALDH^+$ and $CD24^{low}/CD44^{high}$ CSC population in the mammospheres. In contrast, the CSC population in the mammospheres was not affected by Lipo-DS, Cu and anti-BC drugs (Fig. 5.3D and E).




B
T47D CSCs
MCF7 CSCs
- VE
Lipo-DS/Cu
- VE
Lipo-DS/Cu
Bcl2
Bax
Cleaved PARP
α-Tub


C
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Lipo-DS
Cu
Lipo-DS/Cu
MCF7
T47D
Mammosphere number/well
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50
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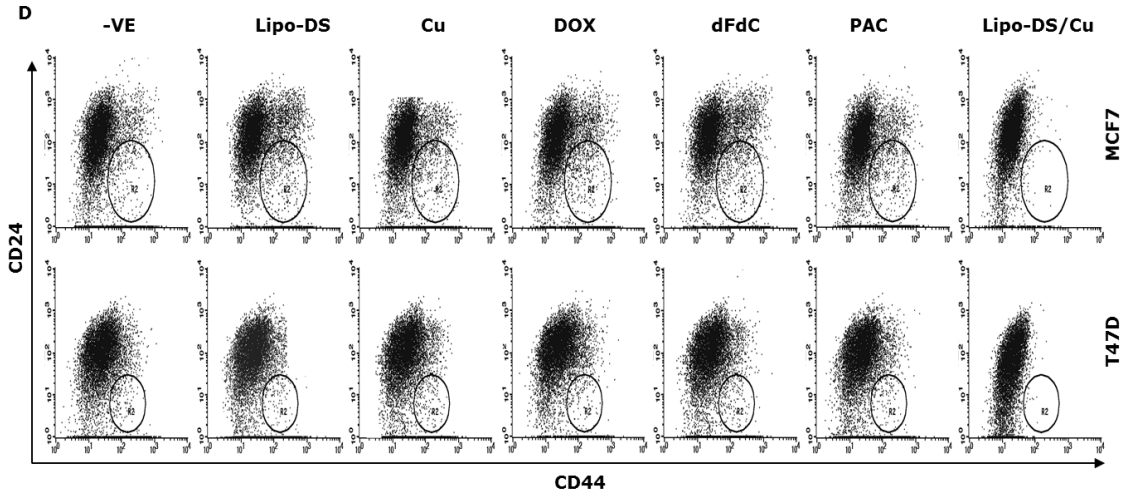
Lipo-DS
Cu
Lipo-DS/Cu
T47D
MCF7


D
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Lipo-DS
Cu
DOX
dFdC
PAC
Lipo-DS/Cu
MCF7
T47D
CD24
CD44

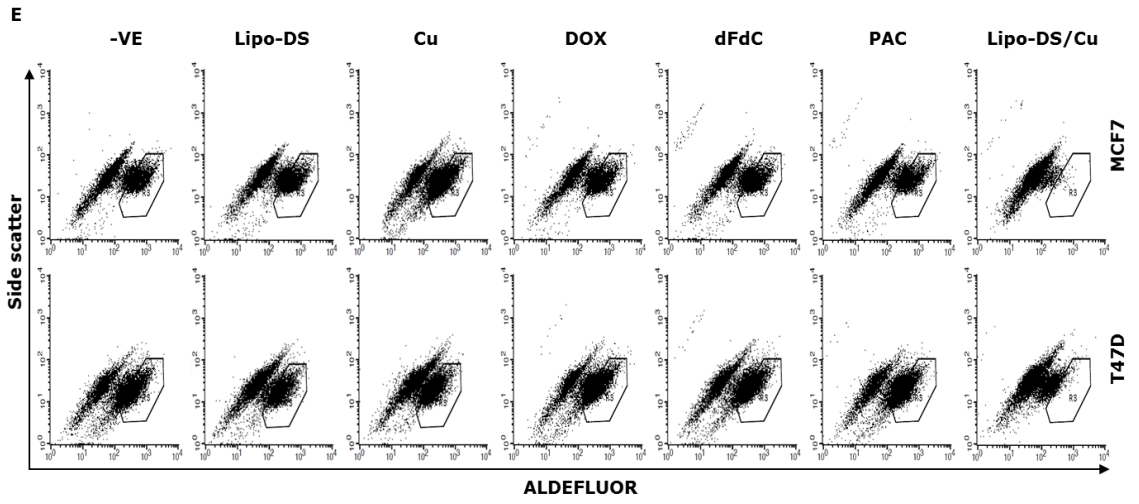


Fig. 5.3 (A-E) Cytotoxic effect of Lipo-disulfiram/copper on breast cancer stem cells. (A) Comparable cytotoxicity of Lipo-DS/Cu in monolayer-cultured cells and BCSCs was detected by MTT assay. The cells were exposed to Lipo-DS/Cu for 72 hours. (B) Lipo-DS/Cu induced BAX and cleaved PARP and reduced Bcl2 expression were detected by western blot. The cells were treated in Lipo-DS (1 μM)/$CuCl_2$ (10 μM) for 4 hours and in drug-free free stem cell culture medium for 24 hours. (C) Lipo-DS/Cu abolished sphere-forming ability in BC cell lines. The cells were cultured in Lipo-DS (1 μM)), $CuCl_2$ (10 μM) or Lipo-DS/$CuCl_2$ for 4 hours and in drug-free free stem cell culture medium for 7 days. (D) The effect of different treatments on $CD24^{low}/CD44^{high}$ population. (E) The effect of different treatments on ALDH activity in BCSCs. For experiments D and E, 7-day-cultured sphere cells were trypsinized and exposed to different agents for 4 hours and released for 24 hours.

Four major ALDH isoenzymes have been detected for response to ALDH activity in CSCs. ALDH1A1 mRNA and protein were not detected in the attached and spheroid cultured cells in both cell lines. In contrast, it was highly expressed in another BC cell line, MDA-MB-231, which we used as the positive control. ALDH2, a mitochondria located isoenzyme, was expressed at low levels and not inducible in CSCs. However, the mRNA

and protein of ALDH1A3 and 3A1 were expressed at very low levels in the attached cells and markedly induced by CSC culture (Fig. 5.3F). This result indicates that ALDH1A3 and 3A1 may contribute to the high ALDH activity in the CSCs derived from MCF7 and T47D cell lines.

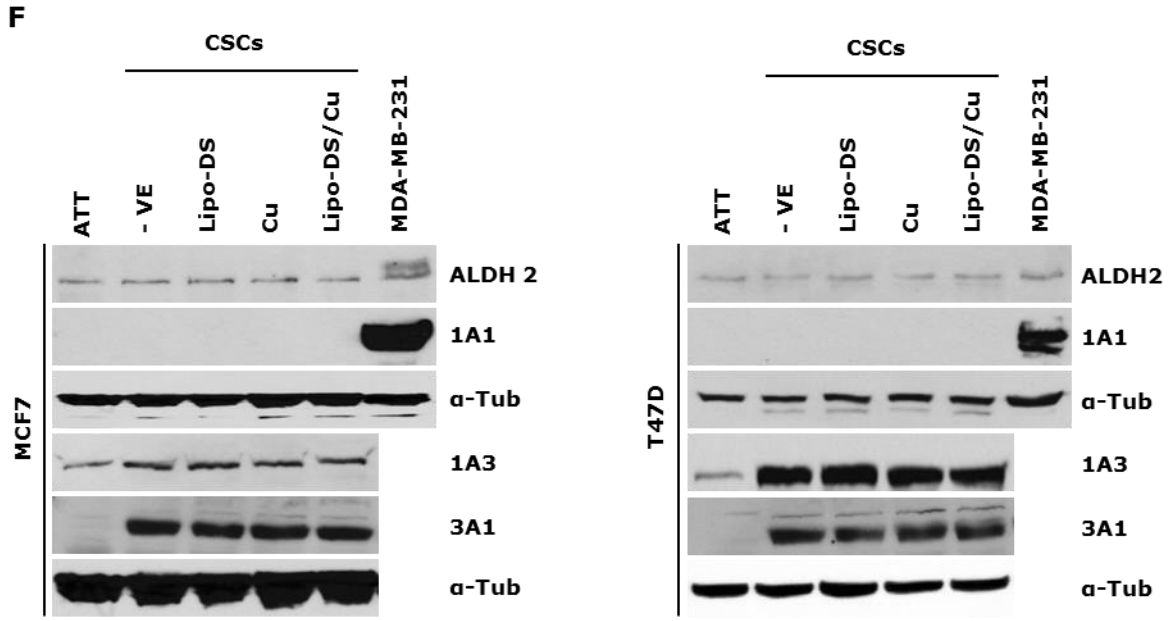
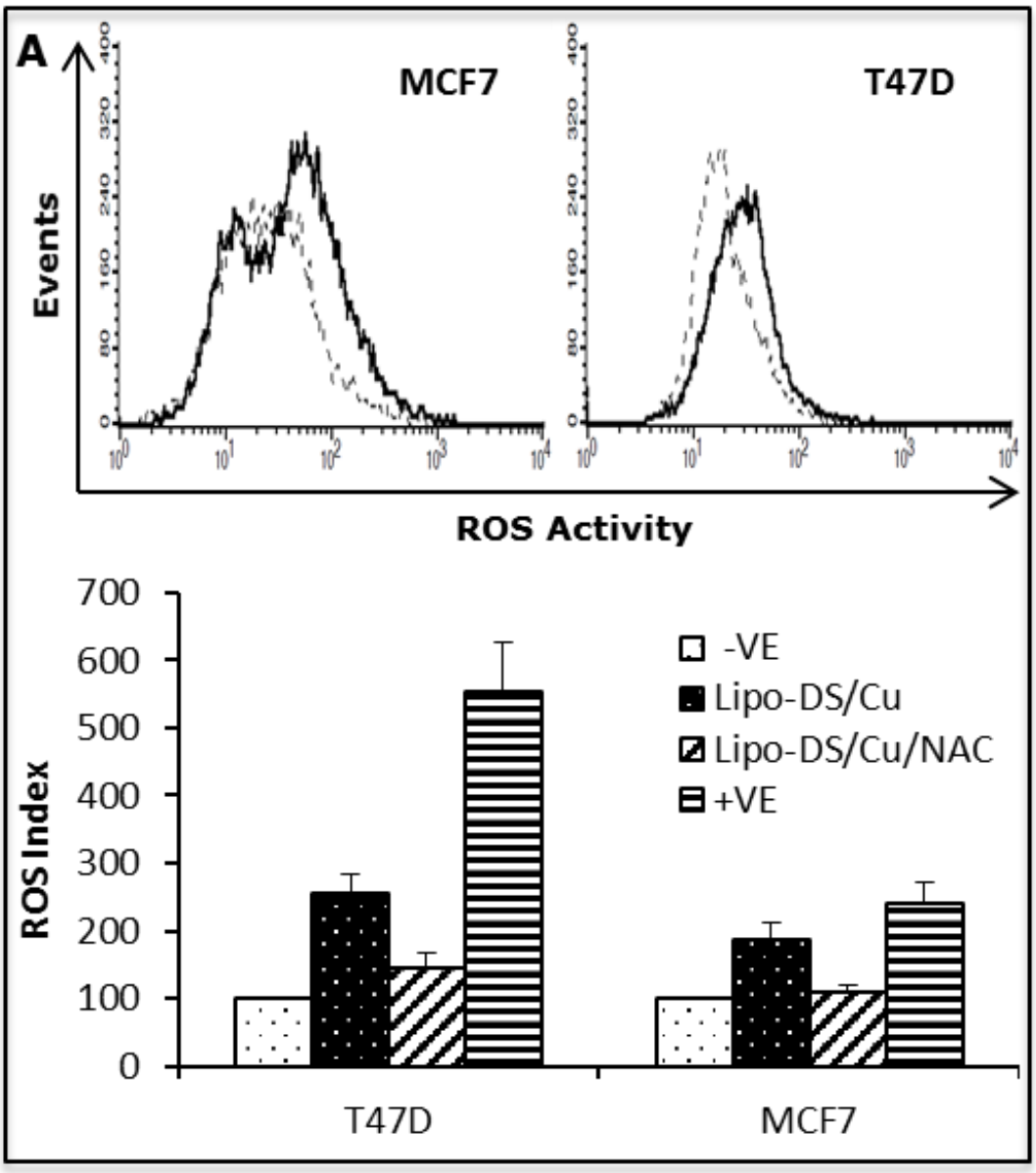


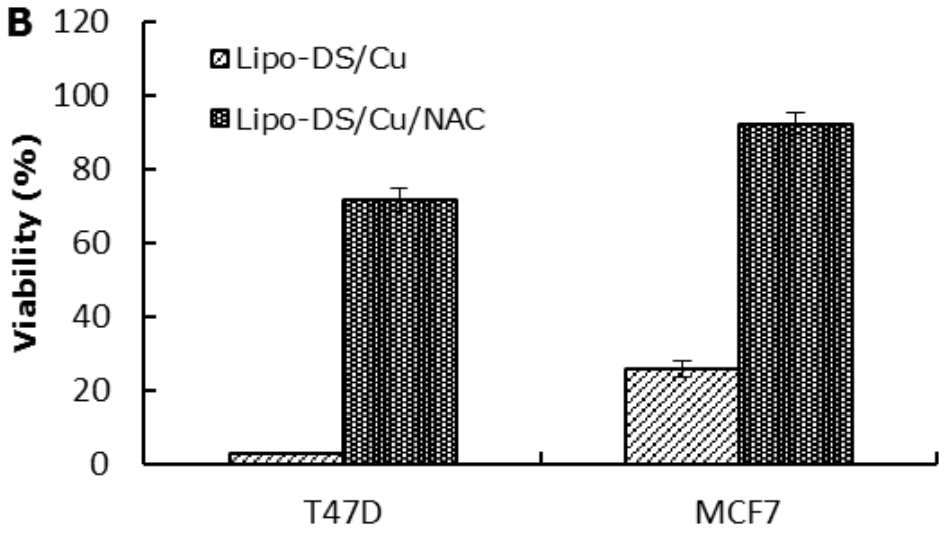
Fig. 5.3F Effect of Lipo-disulfiram/copper on ALDH isoenzymes. (F) Expression of ALDH mRNAs in attached and spheroid cells. The whole proteins were extracted from the BCSCs exposed to different agents for 4 hours and released in drug-free medium for 24 hours.

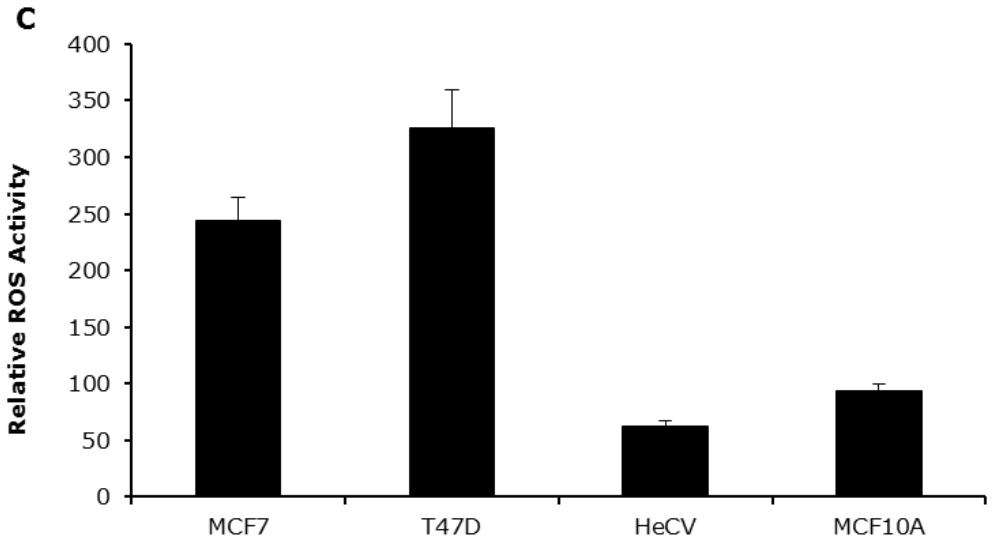
5.3.4 Lipo-Disulfiram simultaneously triggers Reactive Oxygen Species-MAPK activation and inhibits NFκB pathway in cancer stem cells

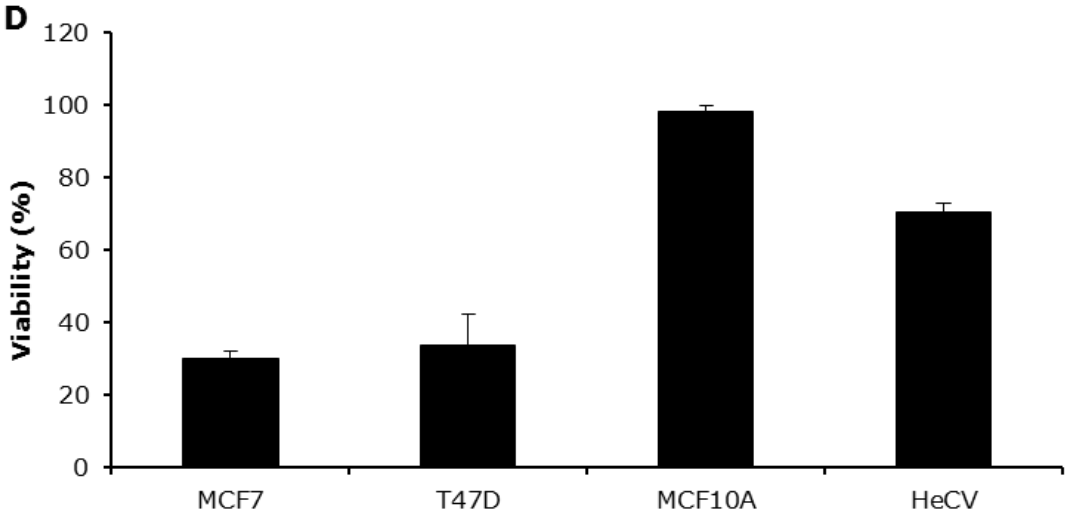
As demonstrated, previous in chapters, DS/Cu activates ROS-MAPK and inhibits NFκB pathways simultaneously in cancer cells. The effect of Lipo-DS/Cu on these pathways in CSCs was examined. Fig.5.4A and B show

that Lipo-DS/Cu treated CSCs generated high ROS activity which was reversed by the ROS inhibitor, NAC. In line with this result, Lipo-DS/Cu induced cytotoxicity was also reversed by NAC (Fig. 5.4C). In comparison with normal cell lines, Lipo-DS/Cu (1 μM/10 μM) selectively induced higher ROS activity and more cell killing in cancer cells (Fig. 5.4D and E).




B
120
100
80
60
40
20
0
Viability (%)
Lipo-DS/Cu
Lipo-DS/Cu/NAC
T47D
MCF7


C
400
350
300
250
200
150
100
50
0
Relative ROS Activity
MCF7
T47D
HeCV
MCF10A


D
120
100
80
60
40
20
0
Viability (%)
MCF7
T47D
MCF10A
HeCV

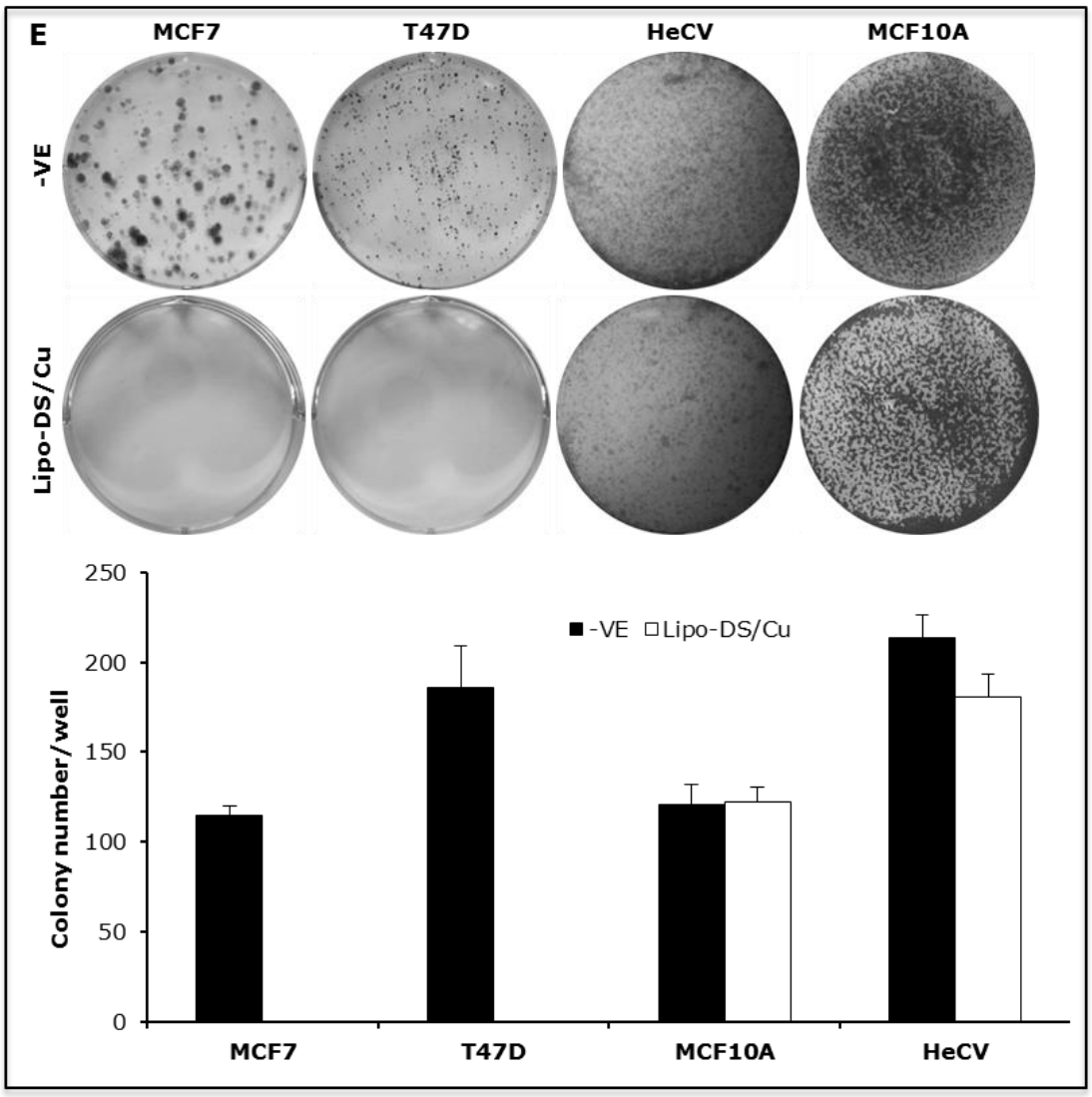
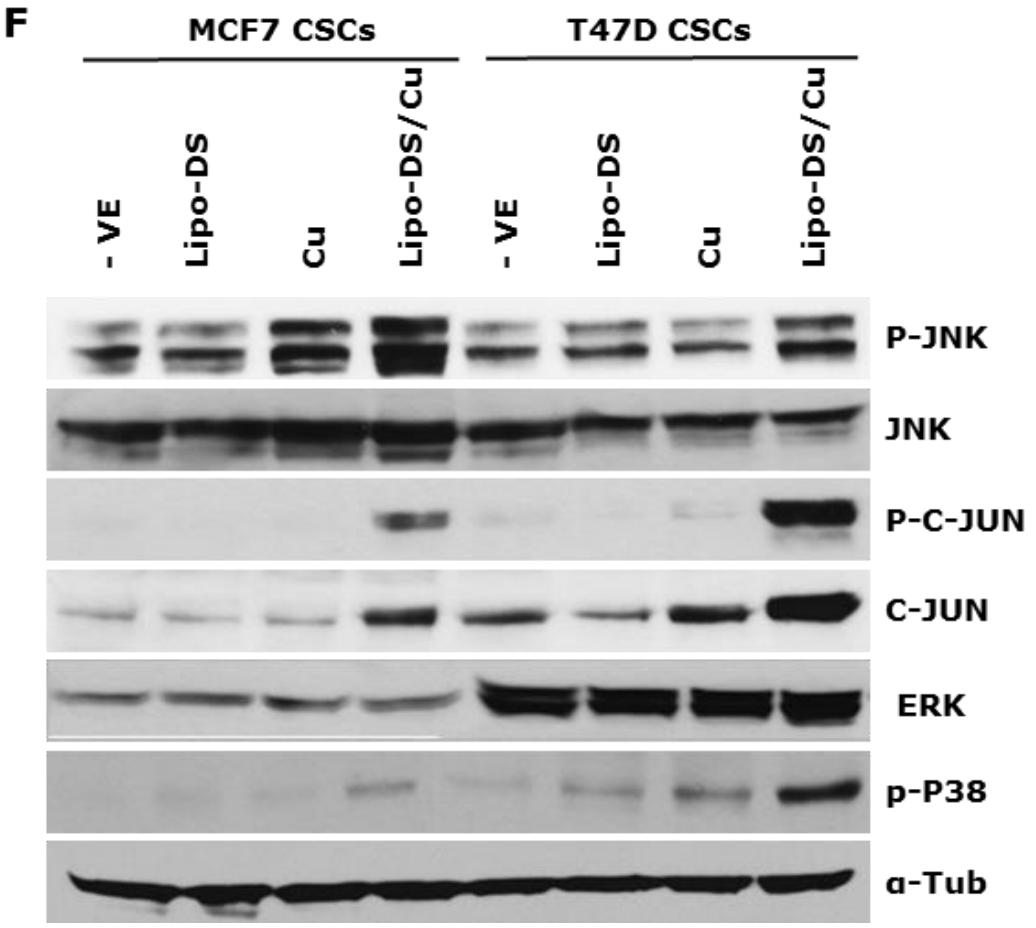


Fig. 5.4 Lipo-DS/Cu induces ROS-MAPK and inhibits NFκB pathways and specifically targets breast cancer cells. (A) Top: Flow cytometric detection of Lipo-DS/Cu-induced ROS activity in BC cells. Dot-line: control, Solid-line: Lipo-DS/Cu. Bottom: Lipo-DS/Cu-induced ROS activity was reversed by NAC. (B) The Lipo-DS/Cu-induced cytotoxicity was reversed by NAC. (C) Lipo-DS/Cu specifically induced ROS activity in BC cell lines. (D) Lipo-DS/Cu demonstrated higher cytotoxic effect on BC than in normal cell lines (MCF10A: Human Mammary Epithelial Cell Line; HeCV: Human Endothelial Cell Line). (E) Clonogenic assay shows that Lipo-DS/Cu abolished colony-forming ability in BC cell lines but had no effect on normal cell lines. The cells were exposed to drugs for 1 hour and released in drug free medium for 72 hours (MTT) and 10 days (clonogenic assay).

Lipo-DS/Cu significantly induced the expression of major MAPK pathway elements (p-JNK, p-c-JUN, c-JUN and p-p38), whereas the ERK expression was not altered (Fig. 5.4F). Furthermore, Lipo-DS/Cu inhibited IκBα degradation, p65 nuclear translocation has been found in both MCF7 and T47D CSCs (Fig. 5.4G). The phosphorylation of NFκB p65 and AKT in the CSCs was also inhibited by Lipo-DS/Cu (Fig. 5.4H).



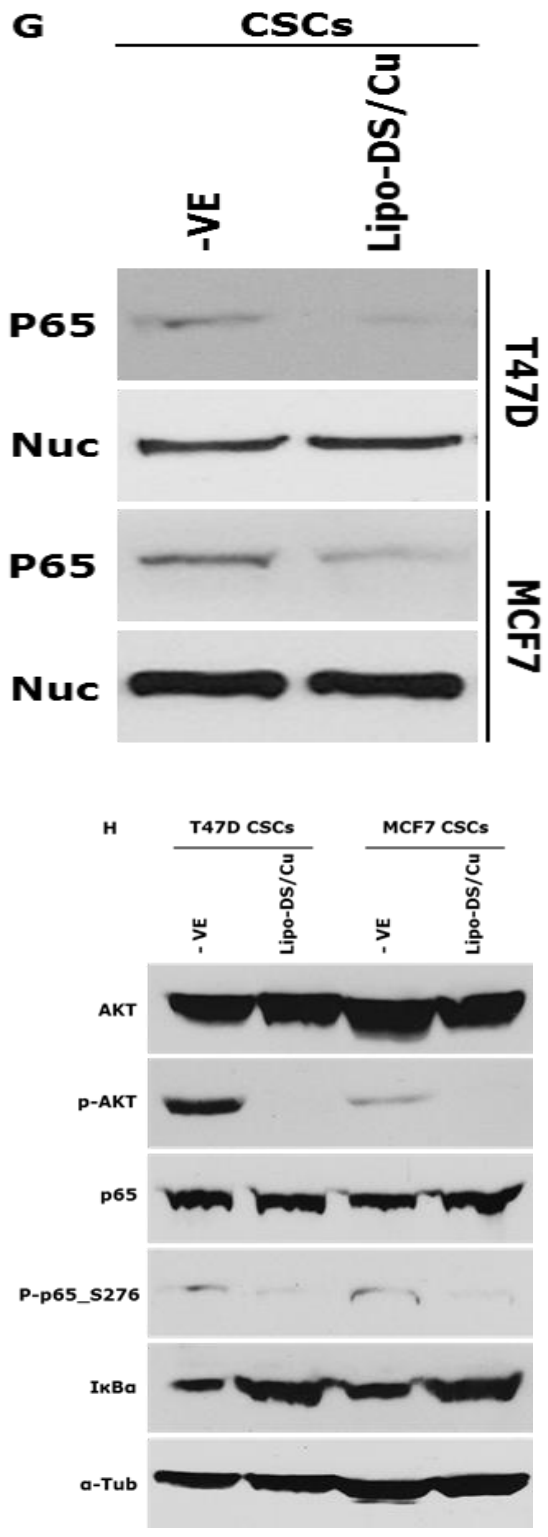
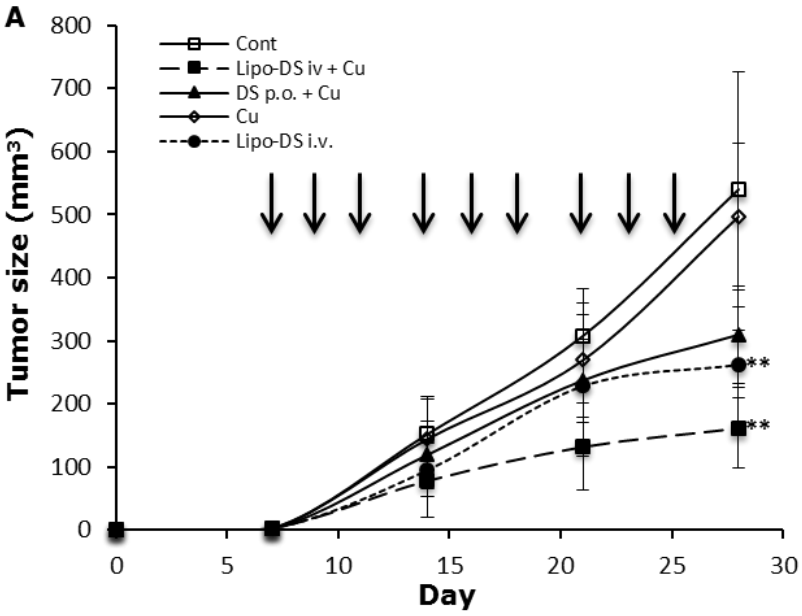


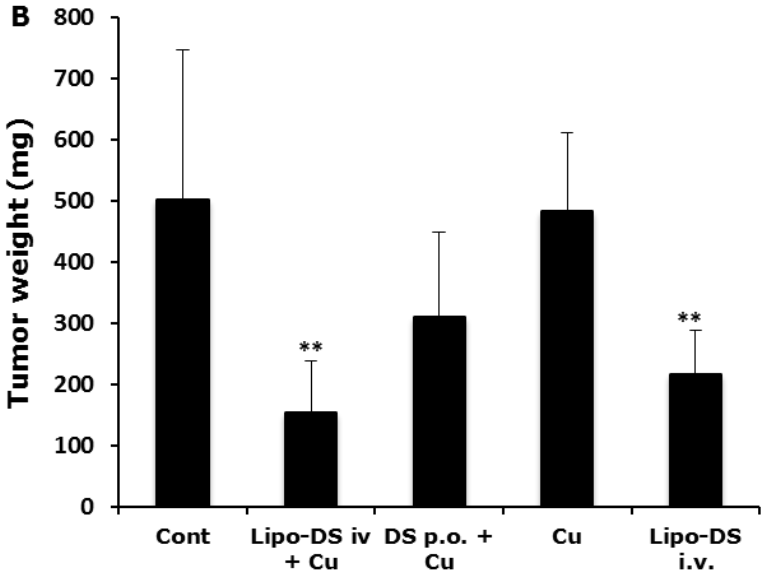
Fig. 5.4 Lipo-disulfiram/copper induces ROS-MAPK and inhibits NFκB pathways and specifically targets breast cancer cells. (F) The effect of different treatments on JNK, p38 and ERK pathways. (G) Lipo-DS/Cu blocked NFκB p65 nuclear translocation. (H) Lipo-DS/Cu induced IκBα expression and inhibited phosphorylation of AKT and NFκB p65. The sphere cells were

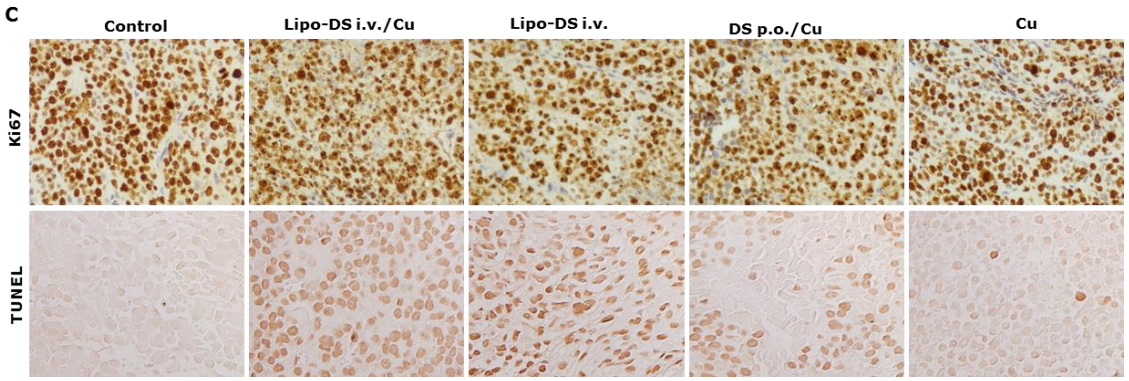
tripsinized and exposed to different treatments (Lipo-DS 1 μM, Cu 10 μM, Lipo-DS/Cu) for 4 hours and released in drug-free medium for 24 hours.

5.3.5 Lipo-disulfiram inhibits breast cancer xenografts *in vivo*

The anticancer efficacy of Lipo-DS has been examined *in vivo*. The dose of Lipo-DS (75 mg/kg) was equivalent to 9 times the human oral dose of 500mg/day. The drug was administered 3 times/week for 3 weeks. The Lipo-DS i.v with/without Cu supplement and Cu alone were compared to determine the necessity of Cu supplement in the anticancer efficacy of Lipo-DS *in vivo*. The oral administration of DS supplemented with Cu was used to compare the anticancer efficacy of different administrative routes. Fig. 5.5A and B show that both Lipo-DS/Cu and Lipo-DS significantly inhibited tumour growth. Oral administration of DS/Cu also inhibited tumour growth although not reaching statistical significance. Cu alone had no effect on tumour growth. Consistent with the *in vitro* data, Lipo-DS induced BAX and inhibited BCL-2 expression, which is in line with the TUNEL result (Fig. 5.5C and D). Furthermore, I examined the effect of Lipo-DS on the vital organs. Fig. 5.5E shows that supplemented with Cu, both oral and intravenous administration of Lipo-DS introduced liver damage. Some necrotic cells were identified in the liver of mice treated with Lipo-DS i.v/Cu or DS p.o/Cu.


A
Cont
Lipo-DS iv + Cu
DS p.o. + Cu
Cu
Lipo-DS i.v.
Tumor size (mm³)
Day
**
**


B
Tumor weight (mg)
**
**
Cont
Lipo-DS iv + Cu
DS p.o. + Cu
Cu
Lipo-DS i.v.


C
Control
Lipo-DS i.v./Cu
Lipo-DS i.v.
DS p.o./Cu
Cu
Ki67
TUNEL

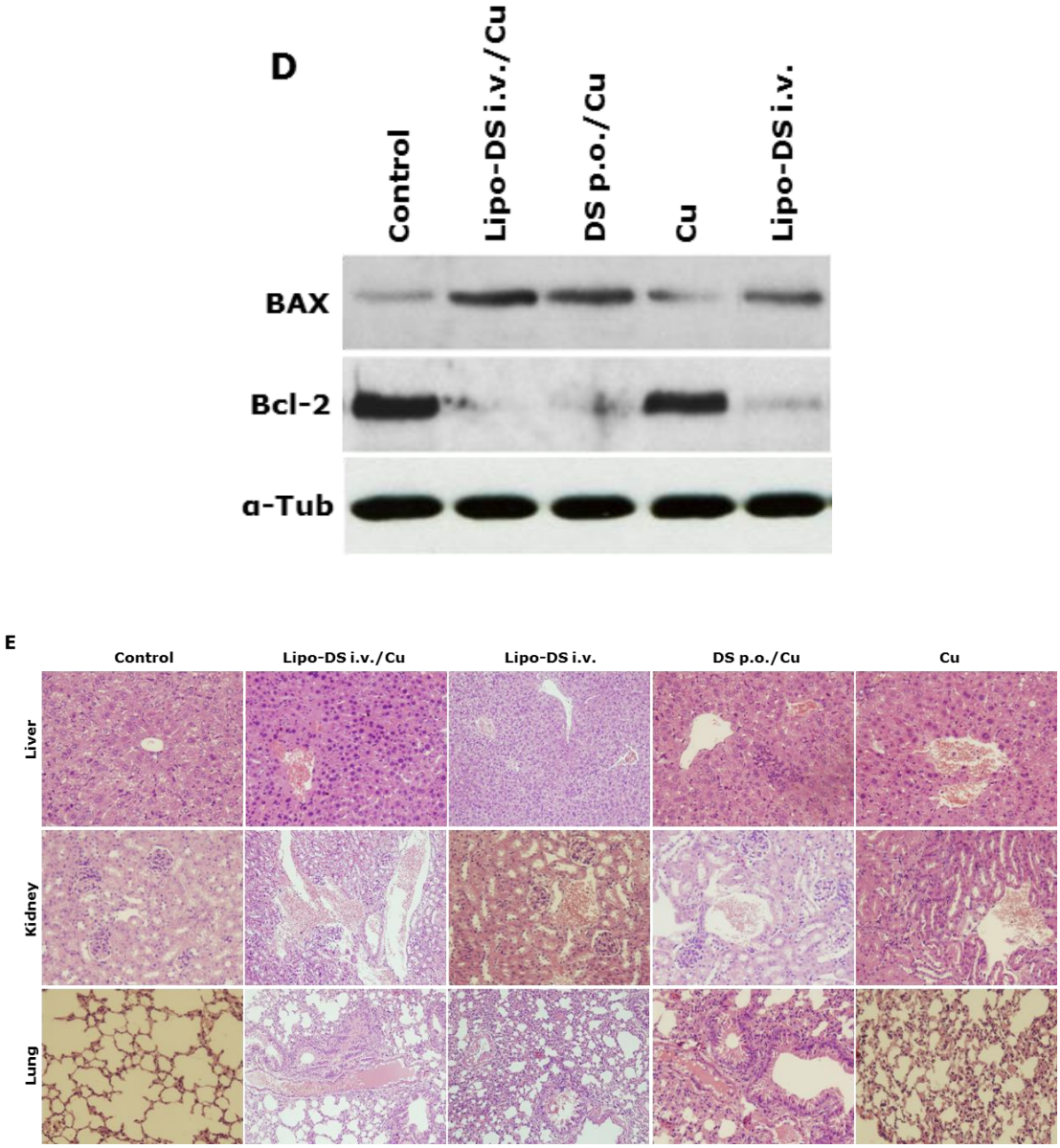


Fig. 5.5 Lipo-disulfiram inhibited growth of breast cancer xenografts. MDA-MB-231 cells (1×10^6) were subcutaneously injected at one rear flank of the mice. When the tumour volume reached ~200mm^3, the tumour bearing mice were randomly subdivided into 5 groups (8 mice/group) e.g. control; DS 75 mg/kg p.o + copper gluconate (CuGlu) 8 mg/kg p.o; CuGlu 8 mg/kg p.o; Lipo-DS 75 mg/kg i.v; Lipo-DS 75 mg/kg i.v + CuGlu 8 mg/kg p.o. The drugs were administered 3 times/week for successive 3 weeks. (A) Growth curves of tumour size. Arrows represent drug administration. (B) The effect of different treatments on tumour weight after 28 days observation. (C) The effect of different treatment on Ki67 and TUNEL expression (×400 magnification). (D) The

effect of different treatments on BAX and BCL2 expression. (E) Histo-pathological images of vital organs (H&E staining, ×400 magnification).

5.4 Discussion

Stem cells possess two unique characteristics: pluripotency and self-renewal, which maintain the organ's structure and function. Recent findings suggest that a small population of cells in tumours behave in a similar manner to normal stem cells (Dick *et al*., 2008). Such cells are CSCs and are considered to be critically important for tumour proliferation, invasion, and metastasis. The concept of CSCs is derived from normal stem cells suggesting that a very small population of cells in cancer bulk possesses stem cell markers. These cells are relatively quiescent, resistant to most anticancer drugs and develop into the original cancer when inoculated into immunodeficient mice. The CSC culture system is adapted from the normal stem cell culture system. In contrast to the normal stem cells, the monolayer-cultured non-CSCs and sphere-cultured CSCs are interconvertible. The essential growth factor-supplemented serum-free spheroid culture has been widely used as a gold method *in vitro* to prevent differentiation, purge the differentiated progenies, and enrich stem cell population (Myant *et al*., 2013). However, the serum-free stem cell culturing system is very expensive and does not very well imitate the physiological *in vivo* conditions. Therefore, I set up BC cell lines in

suspended culture system using both serum-free stem cell medium and serum-rich medium in parallel to determine the necessity of the stem cell medium in maintaining the stemness of CSC *in vitro*. After 5 days culture, the BC cells in both systems formed spheres and clusters (Fig. 5.1A) which represent both cell proliferation and aggregation because if it is not single cell inoculation, the aggregation of the suspended cells is inevitable, no matter how low the density of the seeded cells (Myant *et al*., 2013). The expression of stem cell and embryonic markers was significantly induced in the cells cultured in both systems, even higher in suspension cells (Fig. 5.1B). These results indicated that the stemness is not determined by culture medium, and serum-free stem cell culture system is not essential for CSC culture. Therefore, there must be some common factors introducing and maintaining stemness in the cells cultured in both systems. It is widely accepted that hypoxia is a determinant of stemness in stem cell niche (Zeng *et al*., 2011). I hypothesized that due to the penetrating distance of oxygen, hypoxia may be cumulated in the centre of the sphere which becomes the stem cell niche. Compared with the monolayer-cultured cells, high population of hypoxic cells was detected in the sphere-cultured cells (Fig. 5.1C and D). The spheres developed in serum-containing culture are significantly bigger and tighter than those grown in serum-free culture and contain more hypoxic cells (Fig. 5.1A). In line with the sphere-cultured cells, high population of CSCs was also detected in hypoxia-cultured (<1%

O_2) BC cells. The BC cells cultured in these three conditions are highly resistant to dFdC, Dox and PAC by MTT assay. Chemoresistance is a common feature of CSCs as well as hypoxic cells. These results indicate that the stem cell characters in the sphere-cultured cells may be introduced by hypoxia in the centre of the spheres and the stem cell medium is not necessary for the *in vitro* CSC culture.

Although it is widely observed that hypoxia can induce stem cell characters in normal and CSCs, the detail molecular mechanisms and pathways are still largely unknown. It is reasonable to believe that hypoxia-activated HIF pathways are essential for cellular response to hypoxia. However, many other transcription factors, e.g. NFκB, are also crucial for hypoxia-induced phenotypes. Activation of NFκB pathway has been reported in inflammation-induced hypoxia which enables the survival of the inflammatory cells. Recent studies indicate that NFκB also plays a pivotal role in CSCs, tumour progress and chemoresistance (Zhang *et al*., 2011; Johansson *et al*., 1992). In my study, nuclear translocation of HIF2α and NFκB p65 protein was detected in spheroid and hypoxia cultured cells. These cells also demonstrated IκBα degradation and p65 phosphorylation. The activation of AKT, the transcription factor upstream of NFκB, was also observed. High NFκB DNA binding activity was induced in the cells cultured in all three conditions. In this study, NFκB pathway is activated in

BC cells cultured in all of the three culturing systems (CSC, suspension and hypoxia). In order to examine the importance of NFκB in determination of CSC traits, I have transfected BC cells with NFκB p65. The transfected clones expressed high levels of BCSC markers ($CD24^{low}/CD44^{high}$, $ALDH^{+}$; Fig. 5.2F) and were highly resistant to dFdC, Dox and PAC. These data demonstrate that NFκB transfection definitely conferred CSC traits onto BC cells. The resistant levels of most of the NFκB transfected clones to Dox were lower than those in the spheroid and hypoxic cells. Therefore, some other factors may also be involved in the resistance of Dox in the sphere- and hypoxia-cultured cells.

DS is an NFκB inhibitor and it targets CSCs and reverses chemoresistance in BC cell lines. The application of DS in cancer therapeutics is limited by its very short half-life in the bloodstream (Junttila *et al*., 2008). To overcome this hindrance, we recently developed a Lipo-DS to extend the half-life of DS in the bloodstream (Nakano *et al*., 2006). In line with our previous reports, the cytotoxicity of Lipo-DS is Cu dependent. The cytotoxicity of Lipo-DS/Cu is comparable in both BCSCs and attached cells. After very short exposure (4 hours) to Lipo-DS/Cu, the expression of BCSC markers ($ALDH^{+}$, $CD24^{low}/CD44^{high}$) in the sphere cells was inhibited and the sphere-forming ability in the BC cell lines was completely abolished (Fig. 5.3C). In contrast, the BCSC markers were not affect by

conventional anticancer drug, Lipo-DS or Cu alone (Fig. 5.3D and E). Although Lipo-DS/Cu inhibited ALDH activity, it had no effect on ALDH isoenzymes (1A1, 1A3, 2 and 3A1) at mRNA and protein levels (Fig. 5.3F). It has been reported that ALDEFLOUR detects enzyme activity of ALDH1A1. In this study, I could not detect ALDH1A1 mRNA and protein expression in both cell lines but it is highly expressed in MDA-MB-231 cell line. Therefore, the expression of ALDH1A1 may be cell line dependent. ALDH2, a mitochondrial isoenzyme, was detected at very low basal levels but not induced by spheroid and hypoxic culture. High expression of ALDH1A3 and 3A1 mRNA and proteins was detected in sphere-cultured cells. Therefore ALDH1A3 and 3A1 may be responsible for the high ALDH activity in BCSCs in these two cell lines. A study reported that ALDH1A3 is responsible for the high ALDH activity in BC (Marcato *et al*., 2011).

Selectivity is one of the key issues for anticancer drug development. The cytotoxicity of Lipo-DS/Cu is ROS dependent. ROS damage DNA, RNA and induce apoptosis. Cancer cells possess but are also able to tolerate significantly higher levels of ROS due to the balance of anti-apoptotic mechanisms e.g. NFκB in cancer cells (Margalioth *et al*., 1983). Lipo-DS/Cu selectively induced ROS activity in BC in comparison with normal cell lines and showed significantly higher cytotoxicity in BC than normal

cells. Similar phenomenon was also obtained by clonogenic assay (Fig. 5.4E). ROS induced-apoptosis is highly MAKP pathway-dependent. Lipo-DS/Cu persistently activated JNK, C-Jun and p38 MAPK pathways but had no effect on ERK pathway, which is responsible for cell growth, proliferation and survival (Junttila *et al*., 2008). Apart from activation of pro-apoptotic pathways, ROS also trigger the expression of anti-apoptotic proteins, which neutralize the pro-apoptotic effects of ROS. NFκB is one of the most important ROS-induced transcription factors (Gloire *et al*., 2006). NFκB inhibits JNK and p38 activation by suppressing ROS accumulation in cancer cells. Cancer cell fate is highly dependent on the crosstalk between JNK/p38 and NFκB pathways. The NFκB activity in BC cell lines was significantly inhibited by Lipo-DS/Cu (Fig. 5.4A and B). Simultaneous activation of ROS-JNK/p38 and inhibition of NFκB pathways may contribute to Lipo-DS/Cu induced cytotoxicity in the BC cell lines.

Although Cu is essential for the *in vitro* cytotoxicity of DS, the anticancer efficacy of DS in combination with Cu has never been investigated *in vivo*. In this study, I first tested the anticancer efficacy of free DS and an intravenously applicable Lipo-DS in combination with Cu in BC xenograft. In combination with CuGlu, intravenous administration of Lipo-DS demonstrated the strongest anticancer efficacy. Furthermore, I also examined whether the intrinsic Cu in BC xenograft can enable the

anticancer effect of Lipo-DS. Lipo-DS injection alone also showed strong anticancer activity also it was significantly lower than that of Lipo-DS/Cu combination. Oral version of DS/Cu also showed mild *in vivo* anticancer activity. The Lipo-DS, Lipo-DS/Cu and DS/Cu induced apoptosis was evidenced by TUNEL and western blotting results (Fig. 5.5C and D). In consistence with our *in vitro* data ki67 immunohistochemistry staining indicated that the anticancer effect of DS and Lipo-DS is proliferation-independent. To determine the *in vivo* non-specific toxicity of DS, the pathological changes of vital organs (liver, lung and kidney) in different groups were examined (Fig. 5.5E). Cu induced congestion in major organs. There is no toxicity was observed in the vital organs except the liver in Lipo-DS/Cu treated mice in which some necrotic cells were observed. Our unpublished data indicate that in combination with Cu, the *in vivo* anticancer effect of nano-encapsulated DS could be achieved at significantly lower dose with no toxicity in the liver. Therefore the dose of Lipo-DS in the Lipo-DS/CuGlu still needs to be adjusted.

In conclusion, hypoxia induced NFκB activation is responsible for stemness and chemoresistance in BCSCs. Lipo-DS targets NFκB pathway and CSCs. As a clinically available drug, further study may translate DS into cancer therapeutics.

6. Development of an acquired gemcitabine resistant triple negative breast cancer cell line

6.1 Introduction

dFdC is a nucleoside analogue used for systemic treatment of patients with BC. As the first line drug, dFdC has a high initial activity against tumours, but is induced to acquire resistance later. In clinic, as a single agent, dFdC yields response rates ranging from 14%-37% as first-line therapy for advanced BC and 23%-42% as salvage therapy (Possinger *et al*., 1999). Used in combination with other chemotherapy agents such as PAC and CDDP, dFdC can have even higher response rates. Moreover, the side-effect of dFdC in patients with BC has been favourable compared to other commonly used cytotoxic agents, and the patients present less fatigue, with a notable absence of alopecia and gastrointestinal symptoms. The action of dFdC involves DNA chain termination and mechanisms that result in self-potentiation. The detail of dFdC metabolism and chemoresistance mechanisms have been reviewed in chapter I. Here, I will focus my review on two key factors e.g. deoxycytidine kinase (dCK) and ribonucleotide

reductase subunit 1 (RRM1) playing a pivotal role in the dFdC chemoresistance. dCK is an enzyme, which originates from a family of nucleoside kinases which phosphorylate the 5'-hydroxyl group on (deoxy)ribonucleosides and play a vital role in the salvage pathway. dCK is a rate limiting enzyme for phosphorylation of deoxynucleotides for DNA synthesis. It also activates a number of anticancer and antiviral nucleoside analogues (Ewald *et al*., 2008), particularly dFdC in this study. It has been reported that dCK is involved in cellular response to DNA damage and its activity will be enhanced following treatment with ionising radiation. Thus, this exhibits the potential of dCK activity in radiosensitising effect of dFdC (Pauwels *et al*. 2006). In addition, dCK can inactivate cyclin-dependent kinase 1 (CDK1) which leads to G2/M checkpoint initiation upon DNA damage. This observation is clearly seen in human cervical carcinoma cell line *HeLa* and human embryonic kidney cell line *HEK293T* (Yang *et al*. 2012). Therefore, deficiency in dCK activity has been considered to be one of the main mechanisms responsible for the development of resistance to dFdC. RR is an iron-dependent enzyme and a main element in the DNA replication that catalyses the conversion of ribonucleotide diphosphate to deoxyribonucleotide diphosphate (Kolberg *et al*. 2004). Active RR is a heterotetrameric enzyme composed of two homodimers of nonidentical subunits, RRM1 and RRM2. RRM1 contains the substrate binding and catalytic site as well as the allosteric effector sites while RRM2 contains a

diiron site and a tyrosyl radical that is essential for catalysis (Davidson *et al*., 2004). dFdC inhibits RR activity and blocks the conversion of ribonucleotide diphosphate into deoxyribonucleotide diphosphate which will exhaust the DNA building materials and lead cancer cells into apoptosis. High expression of RRM1 has been observed in dFdC resistant cancer cell lines. The expression levels of RRM1 are also reversely related to the prognosis of non-small-cell lung cancer patients who were subjected to a combinational therapeutics of dFdC and CDDP (Rosell *et al*., 2004; Davidson *et al*., 2004). To date, both dCK and RR have been extensively investigated as potential predictive markers of tumour sensitivity to dFdC (Ueno *et al*., 2007).

Acquired chemoresistant cancer cell lines are important for investigation of the mechanisms of cancer chemoresistance. In this part of my study, I developed and preliminarily characterised a dFdC resistant BC cell line, MDA-MB-231_{GEM100}.

6.2 Methods

General methods have been described in chapter II. The following are the methodologies specifically used in this study.

6.2.1 Cell culture

The dFdC (MDA-MB-231$_{GEM100}$) resistant cell lines were generated from MDA-MB-231 (purchased from ATCC) by being continuously cultured in medium containing dFdC (Sigma, Dorset, UK) in a stepwise concentration-increasing procedure. Firstly, the MDA-MB-231 cells were cultured in medium with dFdC (20 nM) for 4 weeks, then the concentration of dFdC in the culture medium was increased to 50 nM, after 8 weeks cell culture, the MDA-MB-231 cells were cultured in medium containing dFdC (100 nM) for another 8 weeks. The MDA-MB-231$_{GEM100}$ cells were maintained in the medium containing 100 nM of dFdC. All cell lines were cultured in DMEM (Lonza, Wokingham, UK) supplemented with 10% FCS, 50 μg/ml penicillin, and 50 μg/ml streptomycin.

6.3 RESULTS

6.3.1 MDA-MB-231$_{GEM100}$ cells are resistant to gemcitabine

The cytotoxic effect of dFdC on both sensitive and resistant cell lines was compared by MTT assay (Table 6.1 and Fig. 6.1). The IC_{50}_72h of MDA-MB-231 cell line to dFdC is 32.8 nM. In contrast, the MDA-MB-231$_{GEM100}$ cell line is highly resistant to dFdC with an IC_{50}_72h of 91.9 nM.

Table 6.1 Cytotoxicity of dFdC to MDA-MB-231 and MDA-MB-231_{GEM100} breast cancer cell lines

	MDA	MDA_{GEM}
dFdC (nM)	32.8 (13.3)	91.9* (17.9)

The figure represents IC_{50} value from three experiments [mean (SD)]. **p<0.01' *p<0.05 (n=3). The cells were treated for 72 hours.

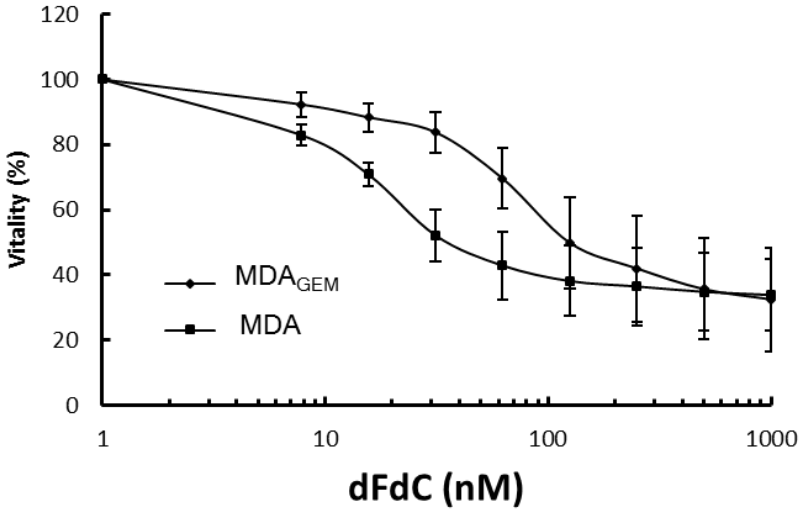


Fig. 6.1 MDA-MB-231_{GEM100} cell line is resistant to dFdC. The MDA-MB-231_{GEM100} and MDA-MB-231 cell lines were exposed to dFdC for 72 hours before MTT assay. MDA_{GEM}: MDA-MB-231_{GEM100}, MDA: MDA-MB-231.

Furthermore the regenerating abilities of resistant and wild type cell lines were examined using clonogenic assay. In line with the MTT data, 30 nM dFdC eliminated the clonogenicity of the parental cell line but no obvious effect on MDA-MB-231_{GEM100} cells (Fig. 6.2). Due to the slower proliferation rate, the colonies developed from the resistant cell line are

smaller than that from the parental cell line. The number of colonies also indicated that in long term DA-MB-231$_{GEM100}$ is also survived from dFdC exposure.

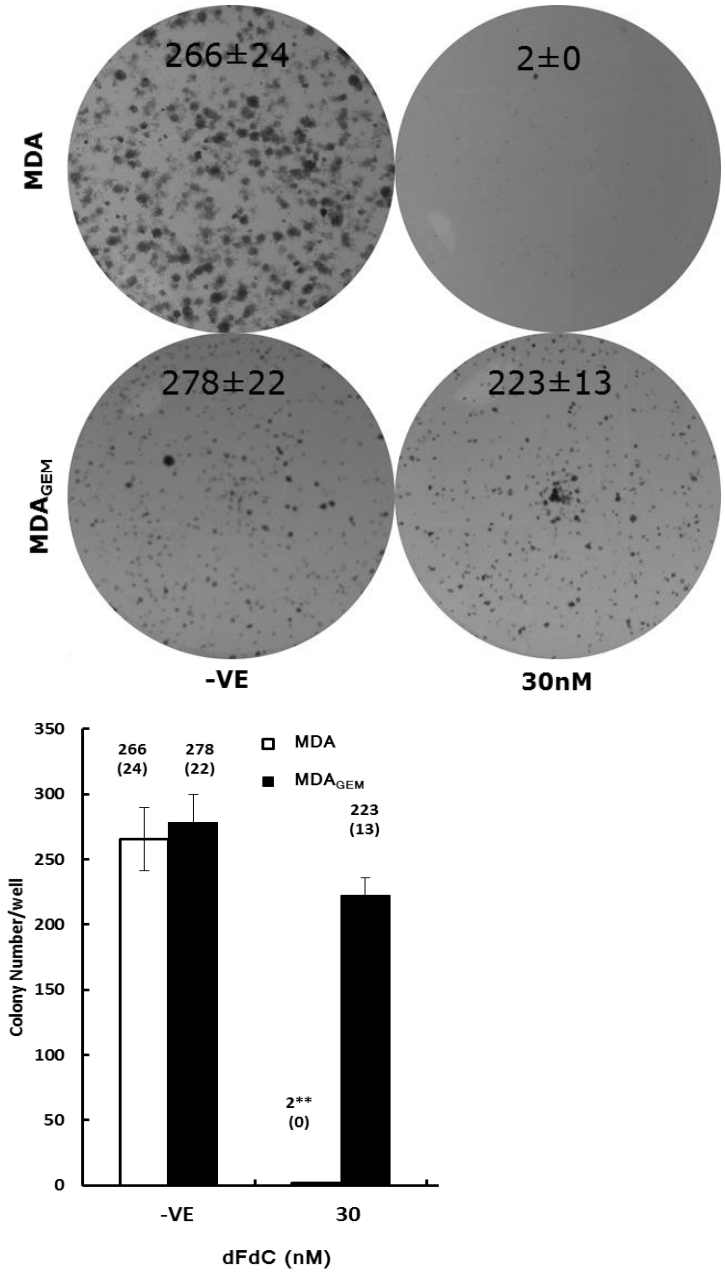


Fig. 6.2 Clonogenic assay of MDA-MB-231$_{GEM100}$ and MDA-MB-231. The cells were exposed to dFdC (30 nM) for 72 hours and then subcultured in drug-free medium at a cell density of 2,500 cells/well in 6-well plates for 10 days. The colonies (≥ 50 cells) grown in each well were counted.

The figures indicate mean (SD). N=3. **p<0.01. MDA: MDA-MB-231, MDA_{GEM}: MDA-MB-231_{GEM100}

6.3.2 Resistance of MDA-MB-231_{GEM100} cell line to gemcitabine-induced apoptosis

After 72h exposure to 30nM dFdC, the phase contrast microscopic images show that sensitive cells undergo apoptosis. In contrast, there was no obvious apoptotic morphological change observed in the resistant cells (Fig. 6.3).

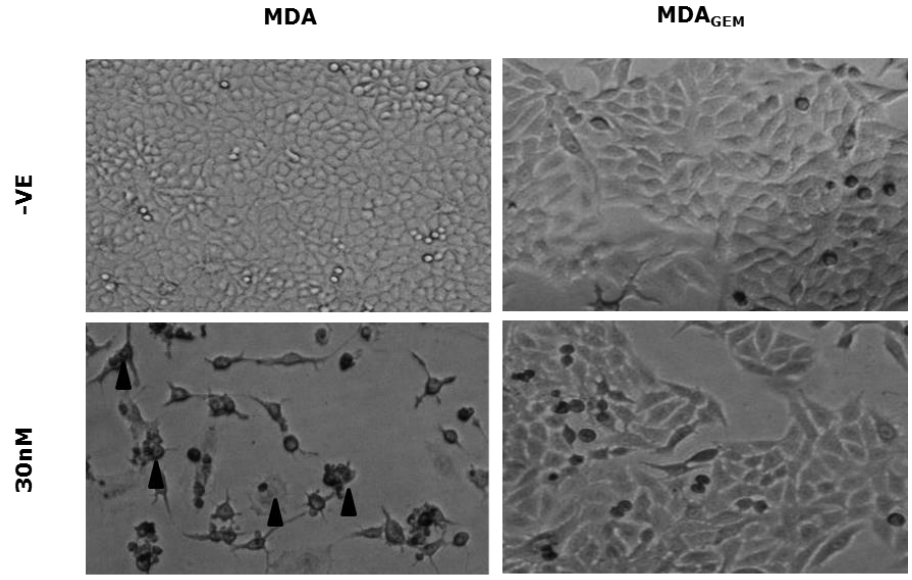
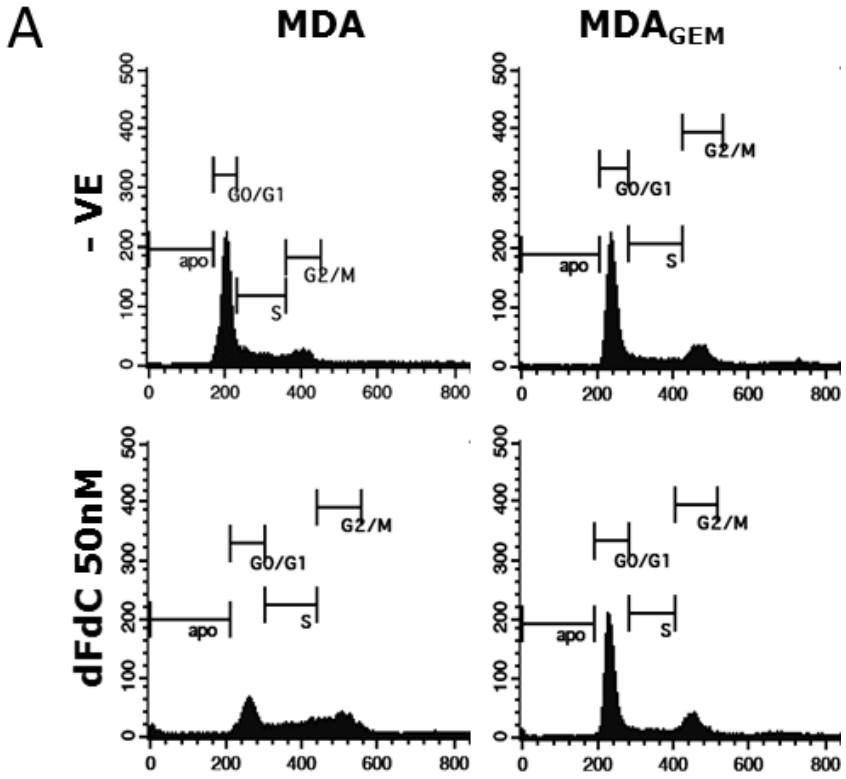


Fig. 6.3 MDA-MB-231_{GEM100} cell line is resistant to gemcitabine-induced apoptosis. The morphology (× 100 magnification) of MDA and MDA_{GEM} cells treated with dFdC (30 nM) for 72 hours. MDA: MDA-MB-231; MDA_{GEM}: MDA-MB-231_{GEM100}

Furthermore the DNA contents in both cell lines were examined by flow cytometry. The cells were treated with dFdC 50 nM for 72 hours and

results showed a significantly higher apoptotic sub-G1 population in the parental cell line than resistant cell line (Fig. 6.4A). The treatment increased S-phase population in the parental cell line. In contrast, there is no significant effect of dFdC on the apoptotic status in the resistant cells. The S-phase cells in MDA-MB-231_{GEM100} cell line have a limited effect by dFdC exposure (Fig. 6.4B and C).



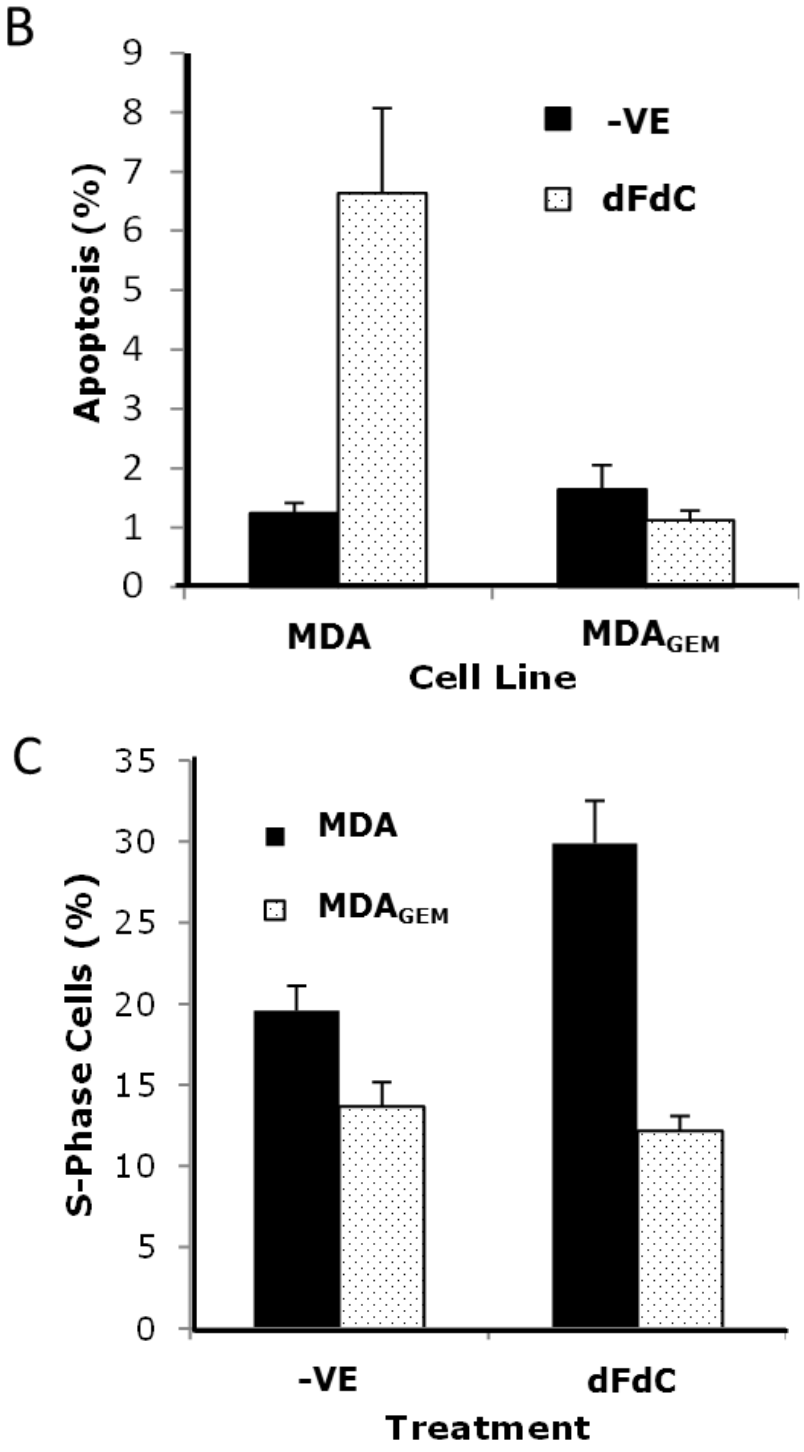


Fig. 6.4 MDA-MB-231$_{GEM100}$ cell line is resistant to gemcitabine-induced apoptosis detected by flow cytometric DNA analysis. (A) Flow cytometric analysis of resistant to dFdC-induced apoptosis in MDA-MB-231$_{GEM100}$ cell line. (B) and (C) The effect of dFdC (50 nM, 72 hours) on cell cycle parameters (Apoptosis and S-phase cells) in MDA-MB-231 and MDA-MB-231$_{GEM100}$ cell lines.

The figures represent the mean (SD) from three experiments. MDA_{GEM}: MDA-MB-231_{GEM100}, MDA: MDA-MB-231.

6.3.3 MDA-MB-231_{GEM100} cell line has a longer doubling time

Quiescence is a common feature of drug resistant cancer cell lines. In my study, I observed that the drug resistant MDA-MB-231_{GEM100} cells grow significantly slower than MDA-MB-231 cells. Therefore I compared the doubling time between these two cell lines. Fig. 6.5 shows the growth curves of both cell lines. The doubling time of MDA-MB-231_{GEM100} cells (81.7h) is significantly longer than that of the sensitive cells (30.8h) ($p<0.01$).

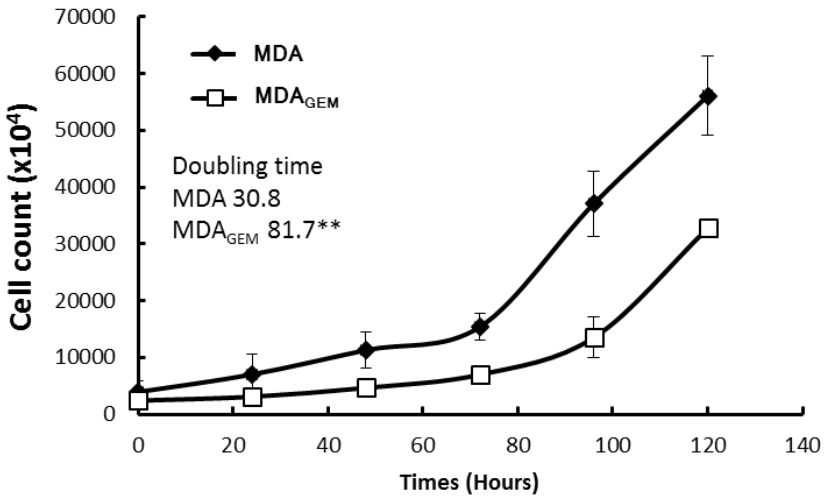


Fig. 6.5 Growth curves of MDA-MB-231 and MDA-MB-231_{GEM100} cells. The cells (5×10^3/well) were cultured in 24-well plates in triplicate. The cells were collected by trypsinization and cell numbers in each of three wells were counted every 24 hours for 120 hours. MDA-MB-231_{GEM100} cell line proliferates slower. MDA: MDA-MB-231, MDA_{GEM}: MDA-MB-231_{GEM100} cells. The figures represent mean of three experiments. **$p<0.01$.

6.3.4 Expression of gemcitabine resistance-related proteins in MDA-MB-231 and MDA-MB-231$_{GEM100}$

It is widely known that deregulation of some key factors, e.g. dCK and RRM1, is involved in dFdC chemoresistance. The expression of dCK and RRM1 were detected in both MDA-MB-231 and MDA-MB-231$_{GEM100}$ cell lines by Western blot (Fig. 6.6). The expression of dCK was considerably low while RRM1 exhibited higher in resistant MDA-MB-231$_{GEM100}$ cell line when compared to sensitive MDA-MB-231 cell line. No expression of Pgp in both MDA-MB-231 and MDA-MB-231$_{GEM100}$ cell lines (Fig. 6.6). Resistant MDA-MB-231$_{PAC10}$ BC cell line, which expresses high levels of Pgp was used as a positive control.

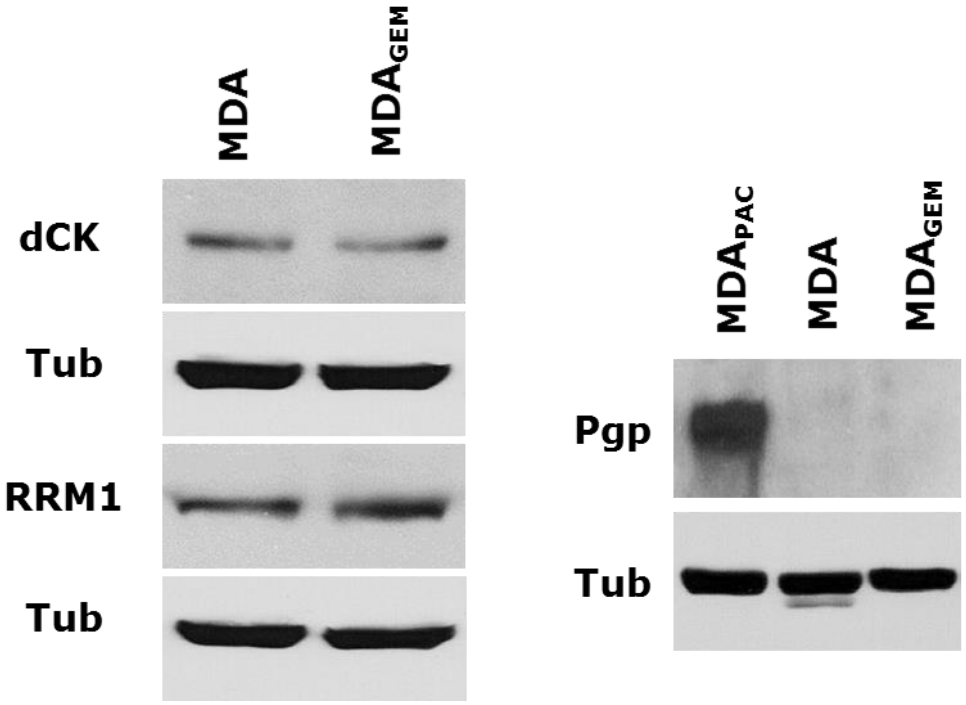


Fig. 6.6 dFdC related protein expression. Western blotting analysis of Pgp, dCK and RRM1 expression. MDA: MDA-MB-231; MDA_{GEM}: MDA-MB-231_{GEM100}; MDA_{PAC}: MDA-MB-231_{PAC10}.

6.4 Discussion

TNBC have poor chemotherapeutic outcomes compared with other BC subtypes. Although the therapeutic outcomes of TNBC have been improved significantly in the recent years, chemoresistance remains the major obstacle for clinical success. dFdC has a wide range of anti-tumour activity against malignancies. dFdC resistance remains vital for the cause of relapsed tumours. The resistant cell line is important for investigation of the mechanisms of dFdC resistance. Therefore I have developed a dFdC resistant TNBC cell line from parental MDA-MB-231 cell line by continuously culturing in medium containing dFdC in a stepwise concentration-increasing procedure. To validate the characteristics of the newly developed dFdC resistant cell line could be contribute to the understanding of chemoresistance in future work.

The *in vitro* cytotoxicity assay revealed that MDA-MB-231_{GEM100} cell line is more highly resistant to dFdC-induced cytotoxicity than sensitive MDA-MB-231 cell line. The IC_{50} of resistant cell line (91.9 nM) is obviously higher than that of sensitive cell line (32.8 nM). In line with the MTT data, clonogenicity of sensitive cell line has almost been abolished by exposure to 30 nM dFdC. In contrast there was no significant cytotoxic effect of

dFdC at the same concentration on resistant cell line. Cell morphology change is also observed; the sensitive cells seemed to shrink and more nuclear degradation. In contrast, dFdC did not induce any apoptotic morphological change in the resistant cell line. All these data indicate that MDA-MB-231$_{GEM100}$ cell line is successfully induced to be resistant to dFdC. The smaller size of colony of resistant cell line is caused by the slower proliferation of resistant cells.

The major obstacle to efficacy of chemotherapy is the development of acquired resistance, which is emerges through unresponsiveness to drugs after an initial success of treatment mainly due to their positioning alteration in the cell cycle. dFdC is an antimetabolite and an S-phase dependent type of drug. Flow cytometric DNA content analysis showed that high population of apoptosis cells in sensitive cell line exposed to dFdC (50 nM, 72h) in contrast with a sharp decrease of apoptosis cells in the resistant cell line. Moreover a high proportion of cells gathered in the S-phase in sensitive cell line treated with dFdC whereas a little bit drop of S-phase cells in resistant cell line. It could be explained that there is a lack of dFdC sensitivity towards resistant cells leading to less apoptotic activity. In addition, the growth curve also showed that the resistant cell line has a longer doubling time with nearly three-folds. These data reflect that why

the cell cycle status does not have significant change after dFdC treatment, suggests dFdC is responsible for S-phase arrest.

Understanding alterations in expression of genes, which characterise the response of cancer cells to dFdC treatment in the process of acquired resistance, would allow us to improve therapeutic strategies for BC. Therefore, some proteins, which may be involved in dFdC resistance, have been examined. In comparison with sensitive cell line, a considerable down-regulation of dCK and up-regulation of RRM1 have been found in the resistant cell line. dCK enzyme plays an important role in conversion of dFdC into its active form and RRM1 is one of the main targets for dFdC (Jordheim *et al*., 2006; Bergman *et al*., 2005). Several reports suggested that there is a link between low expression of dCK and overall survival. One study demonstrated a low OS in patients who had pancreatic adenocarcinoma received dFdC treatment for either metastatic or resected disease had low dCK expression. Similarly, an investigative study on pancreatic cancer with dFdC therapy presents a poor prognosis to patient when dCK expression level is low (Sebastiani *et al*. 2006). RRM1 was known as a primary potential regulatory gene in the pathogenesis of a variety of cancers. RRM1 overexpression is also subjected to a disadvantage in OS. This is evidently verified in RRM1 mRNA expression in advanced NSCLC treated with dFdC and CDDP (Rosell *et al*. 2004). In

addition, high RRM1 expression also could lead to shorter progression-free survival compared to low RRM1 expression in nasopharyngeal carcinoma (NPC) patients treated with dFdC-based chemotherapy (Zhao *et al*. 2012). MDR is known for its unresponsiveness to a variety of chemotherapeutic agents against cancer (Tan *et al*. 2000). Accumulating publications indicated that the main mechanism in cancer cells is increased drug efflux by overexpressing MDR related ABC transporters (Victor *et al*, 1997). One of these commonly discussed among researchers is Pgp. Pgp is used to test in our study but no expression can be found in MDA-MB-231$_{GEM100}$ cell line. However, Pgp is highly overexpressed in resistant MDA-MB-231$_{PAC10}$ cell line due to the presence of a substrate in PAC (Tiwari *et al*. 2009). Based on my data, dFdC resistance is not related to Pgp. It has been reported that the cellular influx of dFdC involves membrane equilibrative (ENT) or concentrative (CNT) nucleoside transporters, such as hENT1, hENT2, hCNT1 and hCNT3 (Shah *et al*., 2007). Due to the time limitation, I did not examine the alteration of these proteins in the resistant cell line in my study.

To summarize, I have characterized a newly developed dFdC-resistant BC cell line, MDA-MB-231$_{GEM100}$, and this cell line is a useful model to investigate the mechanism of dFdC resistance in the BC. Further study on this model will give us hints leading to overcome dFdC related

chemoresistance. My data show that the MDA-MB-231$_{GEM100}$ is more resistant to dFdC and growing slow, low dCK and high RRM1 have been detected in MDA-MB-231$_{GEM100}$. In future, continuing with the current data, I highly recommend my college should examine the cytotoxicity effect of DS on MDA-MB-231$_{GEM100}$. The link between dFdC resistance and BC stem cell could be also investigated as well as the intercellular signalling pathways such as NFκB, ROS-MAPK and stem cell related pathways.

7. General Discussion

Cancer is a serious public health problem worldwide and it is also a major cause of mortality with 28% of all deaths in the UK in 2010. BC is the most common female cancer with the second leading cause of cancer death after lung cancer since 1998 among women in the UK. In the past 30 years, despite the major advances that have been made in understanding the biological and clinical nature of BC, it is still the most prevalent type of cancer among women in the developed world and its incidence has shown a continuous rise in recent decades (Statistical Information Team, CRUK, 2010). Nowadays, BC has become a significant public health problem due to high mortality all over the world. Early stage BC patients with the appropriate treatments have long-term overall survival. Although improvements with better understanding of the use of adjuvant therapies

for early stage BC, the treatment of A/MBC remains a major challenge. Chemotherapy is one of the principal treatments for the A/MBC due to its chemosensitive such that symptom control and prolongation of survival can be achieved. However, response duration remains disappointingly short and long-term survival remains uncommon (Rouzier *et al*., 2005). While chemotherapy is often capable of inducing cell death in tumours and reducing the tumour bulk, many cancer patients experience tumour recurrence and ultimately death because of treatment failure. The major reason for therapeutic failure of patients with A/MBC has been thought to be the drug resistance, which is the main limitation of chemotherapy effectiveness. Several mechanisms have been proposed to implicate in chemoresistance including overexpression of MDR, disfuntion of apoptosis signalling pathways, CSCs, and hypoxic microenvironment in tumour bulk (Moscow *et al*., 1988; Longley *et al*., 2005; Bharadwaj *et al*., 2004; Heinemann *et al*., 1992; Kiyomiya *et al*., 2001). Currently, the demand for a novel anticancer drug is vast and urgent for the cancer patients. The medical need for better cancer therapies is undiminished while drug development is slow and costly, mainly due to the large risk of toxicity of novel molecules. Development of a new drug takes on average 15 years and US$1.5bn with only 5-25% of new oncology drugs in clinical development actually reaching the market (Walker *et al*., 2009). This dilemma has led to a booming interest in repurposing of known drugs into

new use in the USA, Europe and emerging economies. Nowadays over 30,000 drugs have been on market. Taking their derivatives into account, this is a tremendous resource for drug repositioning. Importantly, the safety and pharmacokinetic profiles of these drugs are well established. Repurposing of such drugs would greatly reduce the time and cost for development of a new drug, which is not only beneficial for the public but also profitable for the pharmaceutical companies. As an anti-alcoholism drug used in clinic for over 60 years, the metabolism and clinical pharmacology of DS are well understood. Previous studies indicate its anticancer potential in several different cancer types (Guo *et al*., 2009; Wang *et al*., 2004). Therefore I intend to translate it into cancer therapeutics in my study. My data have demonstrated that DS does not have toxicity to normal cells, but exhibits excellent activity against BC *in vitro*. Importantly, I have also demonstrated that this drug specifically and effectively destroys drug resistant CSCs and reverses chemoresistance. Furthermore, DS potentiates the cytotoxicity of two first-line conventional anti-BC, PAC and dFdC *in vitro (*Yip *et al.*, 2011; Liu *et al.*, 2012*)*. Although DS shows strong anticancer activity in laboratory its application in cancer clinic is highly limited by its bio-instability. The half-life of DS in the blood stream is only ~4 minutes (Cobby *et al*., 1977). Clinical translation of DS as an anti-cancer drug is limited by its rapid degradation and extensive metabolic conversion. While the DS metabolites are active

against alcoholism, the anti-cancer activity requires unmodified DS. Therefore, in order to translate DS into cancer therapeutics, we need an efficacious delivery system to protect DS in bloodstream and transport it into cancer tissues. Our collaborators in Shenyang Pharmaceutical University, China encapsulated DS into liposome and extend the half-life of DS in the bloodstream to about 2 hours. The liposomal DS (Lipo-DS) improved the anticancer efficacy of DS in a mouse model.

Cu is an essential trace element that plays a critical role in oxidation-reduction (redox) reactions and in scavenging free radicals (Turnlund *et al*., 1998). As early as 400 B.C., Hippocrates prescribed Cu compounds to treat diseases (Turnlund *et al*., 2006), and the successful animal experiment in cancer treatment was published almost 9 decades ago (Hieger *et al*., 1926), whereas scientists are still uncovering new information regarding the functions of Cu in the human body (Prohaska *et al*., 2012). In the body, Cu shifts between the cuprous (Cu^{1+}) and cupric (Cu^{2+}) forms, and the majority is $Cu2^{+}$ form. Based on my results, single Cu treatment does not show any cytotoxicity to the BC cell lines. The intracellular transport of Cu is still one of the major hurdles for its clinical efficacy due to the complicated environment in the cancer cells. Ctr1 is a Cu transporter, responsible for the transport of Cu into cells. In order to maintain redox homeostasis, intracellular Cu levels are strictly controlled by Ctr1 (Kim *et al*., 2008).

The anticancer activity of DS is $Cu2^{+}$ dependent. DS strongly chelates Cu to form a DS/Cu complex. Cancer tissues possess significantly higher levels of Cu than their normal counterparts. This statement will give DS the selectivity to target cancer cells. The potential explanation of Cu-induced cellular toxicity is that Cu ions participate in the formation of ROS. Cu induces the generation of hydroxyl radicals (OH^{-}) from H_2O_2. The hydroxyl radical is the most powerful oxidizing radical as it is theoretically capable of reacting with every biological molecule and thus initiates oxidative damage. As a strong bivalent metal ion chelator, DS and its derivative, N,N-diethyldithiocarbamate (deDTC), can form a complex [Cu(deDTC)2] with Cu. This DS/Cu complex can transport Cu into cancer cells in a Ctr1-independent manner thus overcoming the transporter-controlled regulation of intracellular Cu homeostasis (Cen *et al*., 2004). My results indicate that the cytotoxicity of DS/Cu in BC is ROS-related. DS/Cu induced ROS activity and cytotoxicity was reversed by NAC, an inhibitor of ROS. DS/Cu persistently activated JNK and p38 MAPK pathways that promote ROS-induced apoptosis in both cancers (Junttila *et al*., 2008). In my study, I have examined different time points for the treatment of DS/Cu in BC (1, 8, 24 hours) cell lines, the results showed that both JNK and p38 pathways were persistently activated (phosphorylation of c-Jun and p38) by DS/Cu and blocked by NAC. In contrast, DS/Cu did not activate the ERK pathway, which plays an essential role in cell growth, proliferation and survival

downstream of ROS. ROS-activated JNK and p38 pathways were partially responsible for ROS-induced apoptosis because the inhibitors of JNK and p38 reduced cytotoxicity of DS/Cu but to a lesser degree than ROS inhibition. It widely known that the activation of JNK and p38 induces apoptosis via mitochondrial apoptotic pathways (Junttila *et al*., 2008). Pro-apoptotic BAX was induced and anti-apoptotic BCL2 was inhibited by DS/Cu leading to an increased BAX/BCL2 ratio and thus a pro-apoptotic phenotype in response to ROS. These results indicate that DS/Cu may trigger intrinsic apoptosis via persistent activation of JNK and p38 pathways that is ROS-dependent. NFκB is one of the most important ROS-induced anti-apoptotic factors (Gloire *et al*., 2006). The activation of NFκB in turn inhibits ROS and JNK, p38 activation and ultimately inhibits ROS-induced apoptosis. Cancer cell fate is highly dependent on the crosstalk between JNK/p38 and NFκB pathways. My data showed that both the binding activity and transcriptional activity of NFκB were significantly inhibited by DS/Cu in BC cell lines, and ultimately induce the cells to undergo apoptosis. To date, one of the possible cytotoxicity mechanisms of DS/Cu is simultaneous inducing ROS generation to activate ROS-MAPK pathway and inhibiting NFκB pathway.

The most important and novel finding in my study has been thought that DS can reverse the chemoresistance by targeting CSC. CSCs are

considered to be a very small population of cancer cells that can have unlimited growth, self-renewal, as well as differentiation into other, more specialized cancer cell types (Ichim *et al*., 2006). CSCs have been implicated in the initiation and development of malignancy. Many cases demonstrated that CSCs are able to survive and regenerate the tumour even after a large percentage of malignant tissue has been destroyed by chemotherapy, and this has been considered as one of the major causes of chemoresistance, which ultimately leads to the cancer relapse. My results showed that DS in combination with Cu could target CSC to reverse the chemoresistance and prevent the tumour recurrence. The first evidence is from MDA-MB-231$_{PAC10}$, a cell line resistant to PAC. Cancer cells displaying reduced sensitivity to drugs through continued exposure can also become cross-resistant to other unrelated classes of chemotherapeutic drugs. The pan-resistance is the major obstacle to successful chemotherapy. Inadequate drug exposure and insufficient dosage are the main reasons to induce the multidrug resistance during chemotherapy. MDA-MB-231$_{PAC10}$ resistant cell lines were generated from MDA-MB-231 by being continuously cultured in medium containing PAC (10 nM) in a stepwise concentration-increasing procedure. A few different chemotherapy drugs share a common transport mechanism, which is controlled by ABC transporter family. As a member of ABC transporter family, high expression of the MDR1 gene in multidrug resistant cancer cells leads to a

large production of MDR1 protein, which is commonly known as Pgp. It pumps out anti-cancer drug from the cytosol to the extracellular medium by using the energy of ATP hydrolysis (Moscow *et al*., 1988; Gottesman *et al*., 1993). High expression of Pgp is also a common feature of CSCs. My results showed that MDA-MB-231$_{PAC10}$ cell line shows high expression of Pgp protein and the cell cycle of MDA-MB-231$_{PAC10}$ is slower than the sensitive cell line. These results suggest that this cell line may contain high population of CSCs, which may play a key role in PAC resistance. MTT results showed that DS/Cu is highly cytotoxicity to both sensitive and resistant cell lines. The CSC markers in this resistant cell line were also inhibited by DS/Cu. DS/Cu could reverse the PAC-induced chemoresistance by targeting CSC. Furthermore, I have examined the effect of DS/Cu on several CSC phenotypes in different cell lines in my study. Accumulating evidence indicates that ALDH may not only be a surrogate marker but also a functional target for anti-CSC chemotherapy. High levels of ALDH were detected in the resistant cells, the similar high activity of ALDH has been found in BC stem cells. It is believed that all three stemness factors (Nanog, Oct4 and Sox2) play a crucial role in the survival of a population of CSCs with drug resistance phenotype (Linn *et al*., 2010). In my study, the embryonic stem cell-associated genes Sox2, Oct4 and Nanog also showed up-regulation in resistant cell line as well as BC stem cells. Meanwhile, high activities of CD44+/CD24- and CD133+

have been detected in BC stem cells. Inhibition of ALDH activity by DS/Cu was confirmed by ALDEFLOUR analysis in BC stem cells, as well as resistant cell line. Further study on the ALDH showed that DS/Cu had no effect on ALDH isoenzymes (1A1, 1A3, 2 and 3A1) at mRNA and protein levels. Although it has been reported that ALDEFLOUR detects enzyme activity of ALDH1A1, no ALDH1A1 mRNA and protein expression can be detected in BC stem cells whereas they are highly expressed in MDA-MB-231 cell line. Therefore, the expression of ALDH1A1 may be cell line dependent. ALDH2 was detected at very low basal levels in BC stem cells. High expression of ALDH1A3 and 3A1 mRNA and proteins was detected in BC stem cells. ALDH1A3 and 3A1 may be responsible for the high ALDH activity. A study reported that ALDH1A3 is responsible for the high ALDH activity in BC (Marcato *et al*., 2011). In addition, DS/Cu has markedly suppressed the stem cell markers CD44+/CD24- and CD133+ in BC stem cells. The expression of Nanog, Sox2 and Otc4 in BC stem cells and resistant cell line has been significantly inhibited by DS/Cu exposure. Overall, CSC markers are not only highly relevant for studies on the biology of CSC, but could also serve as useful markers to monitor the efficacy of differentiation therapy and may have a potential role to be targeted for the cancer therapy. However, the cellular cytotoxicity effect of DS/Cu on CSC is still not fully understood.

My study elucidates the possible mechanistic roles of DS/Cu in targeting BCSCs and reversion of chemoresistance.

It has been proposed that specific therapies against CSCs may lead to the development of curative strategies. New therapies should eradicate the minority population of CSCs, instead of the rapidly dividing but terminally differentiated cells, which constitute the bulk of the tumour. It is predictable that the specific targeting CSCs population should result in better control of the disease. The small molecules may be usefully applied for anti-CSC therapies. These approaches include the targeting of CSC via developmental stem cell pathways and targeting CSC via CSC microenvironment. In my study, DS plus Cu has been used as a bullet to target CSC. The NFκB pathway and hypoxia have been considered to play the key role in the CSC-related chemoresistance process (Scortegagna *et al*., 2008). Firstly, in order to confirm if the CSC features can be also induced by hypoxia, I have cultured monolayer BC cells under hypoxic condition (O_2<1%). In line with the spheres-cultured cells, high population of CSC have been detected in the hypoxic cells. The CSC markers have shown significant increase in both hypoxic monolayer cells and spheres-cultured cells, including ALDH, $CD44^+/CD24^-$, and the expression of stem cell and embryonic markers (Nanog, Sox2 and Oct4) was significantly induced in the cells cultured in both systems. Moreover, hypoxia is a determinant of

stemness in stem cell niche. Due to the penetrating distance of oxygen, hypoxia may be cumulated in the centre of the sphere. Confocal microscopy images showed that compared with the monolayer-cultured hypoxic cells, high population of hypoxic cells was detected in the sphere-cultured cells. The most important finding is that both hypoxic monolayer cells and sphere-cultured cells are highly resistant to PAC, dFdC and Dox. Chemoresistance is a common feature of CSCs. These results indicate that the stem cell characters in the sphere-cultured cells may be introduced by hypoxia. The detail molecular mechanisms and pathways linking hypoxia and CSCs are still largely unknown. It is generally believed that hypoxia-activated HIF pathways are important for cellular response to hypoxia (Raval *et al*., 2005). Moreover, as the most important transcription factors, NFκB is also crucial for hypoxia-induced phenotypes. As I have mentioned, NFκB also plays a pivotal role in CSCs, tumour progress and chemoresistance (Zhang *et al*., 2011; Johansson *et al*., 1992). In my study, nuclear translocation of HIF2α and NFκB p65 protein was detected in both hypoxic monolayer cells and spheres cells. The activation of AKT was detected and IκBα degradation and p65 phosphorylation have been found in these cells. Moreover, the NFκB DNA binding activity was also induced in hypoxic monolayer cells and spheres cells. Therefore NFκB pathway is activated in CSC and hypoxic cells. This suggests that NFκB show its

importance of NFκB in determination of CSC traits. In order to confirm this, I transfected BC cells with NFκB p65. The transfected clones expressed high levels of stem cell markers and were highly resistant to dFdC, Dox and PAC. Therefore NFκB definitely conferred CSC traits onto BC cells. DS is an NFκB inhibitor targeting CSCs and reversing chemoresistance. The cytotoxicity of DS/Cu is comparable in both BC stem cells and attached cells. DS/Cu has inhibited all the CSC markers, but these markers were not affected by conventional anticancer drug. As the similar manner in BC cell lines, the cytotoxicity of DS/Cu is ROS dependent in BC stem cells. DS/Cu selectively induced ROS activity and showed significantly higher cytotoxicity in BC stem cells than in normal cell lines. In line with the previous data, ROS induced-apoptosis by DS/Cu is highly MAKP pathway-dependent with JNK, C-Jun and p38 increase but no effect on ERK. NFκB is one of the most important ROS-induced anti-apoptotic factors. Moreover NFκB inhibits JNK and p38 activation and suppresses ROS accumulation in cancer cells (Gloire *et al*., 2006; Nakano *et al*., 2006). Simultaneous activation of ROS-JNK/p38 and inhibition of NFκB pathway may contribute to DS/Cu induced cytotoxicity in the BC stem cells.

In summary, DS/Cu is not only highly cytotoxic to BC, but can also reverse chemoresistance by targeting CSC. DS shows very strong anticancer activity *in vitro* but free DS is always easily degraded in the bloodstream

before they reach the tumour in the body. Our partners encapsulated the free DS into Lipo-DS and further study on the effect of Lipo-DS in BC xenografts *in vivo.* The animal study results showed that Lipo-DS/CuGlu injection has the strongest anticancer efficacy. Lipo-DS inhibited the $ALDH^+$ CSC population in the xenografts. In order to examine the selectivity of DS *in vivo,* the results of the histo-pathological changes of vital organs (liver, lung and kidney) show no toxicity in the vital organs except in some necrotic cells in the liver of Lipo-DS/Cu treated mice. To date, as a well-understood, safe drug that has been used in anti-alcoholism clinic for over 60 years, DS does not have toxicity to normal cells but demonstrates excellent activity against a wide range of cancers, including the most aggressive form of colon, breast, and brain cancer *in vivo* and *in vitro*. Importantly, this drug specifically and effectively destroys drug resistant CSCs and reverses chemoresistance.

In future, in order to reduce the dose of DS *in vivo,* nano-encapsulated DS could be used for the next stage of research. Novel combination therapies targeting different parts of the tumour microenvironment are critical in order to completely eradicate drug-resistant cancer cells and CSCs. Nanomedicine significantly extends the range of existing anticancer drugs and treatment strategies and can be useful in targeting CSCs. Nanotechnology-based drug delivery system (NDDS) is a rapidly evolving

and expanding interdisciplinary field involved in an amalgamation of chemistry, engineering, biology and medicine. Nanoparticles (NPs) have unique advantages in delivering anticancer drugs to tumour sites and changing the pharmacokinetics and biodistribution of drugs, both of which result in higher antitumour efficiency while reducing side effects. Currently available NPs in cancer therapeutics include dendrimers, liposomes, lipid NPs, polymeric NPs, micelles, gold NPs and protein NPs etc. These NPs can encapsulate and deliver intact drug to cancer tissues. We hypothesized that encapsulation of DS into long circulating nano-materials will improve the anticancer efficacy of DS and finally translate it into clinical trials. In the proposed study, we will further reformulate the DS using different types of nanomaterials, and perform a series of *in vitro* and *in vivo* experiments to explore several features of these novel nanoparticles including their biodistribution, long-term fate, toxicity and antitumour efficacy. Also, the detailed mechanism of DS release from these nanoparticles and how these DSF-loaded nanoparticles execute their antitumour activity will be studied. More importantly, the effect of these nano-encapsulated DS on different cancer models will be examined to evaluate the targeted therapeutic effects. The ultimate goal is to select the best formulation of DS (highest antitumour efficacy without toxicities) for further translational study. Through the effort of a team of researchers comprising nanotechnology engineers, chemists, biologists and oncologists

from different countries, it will be possible to develop DSF-nanoparticles that are both effective and safe, and can thus move a step closer towards human trials.

Furthermore, in line with the exciting data of DS in BC cell lines, I have demonstrated that DS/Cu enhances the cytotoxicity of dFdC in glioblastoma multiforme (GBM) cell lines. Both dFdC and DS can traverse the BBB and get into GBM tissue. The synergistic effect between DS/Cu and dFdC may help to overcome *de novo* dFdC resistance in GBM cells and be beneficial for dFdC-based chemotherapy in GBM patients. My study also indicates that DS/Cu alone or in combination with dFdC may be a successful therapeutic for chemoresistant GBM by successfully targeting chemoresistant CSC via ROS generation and attenuation of ALDH mediated protection from oxidative damage (See the attached paper in Appendix). I highly recommend further study to investigate the opportunity of using DS in GBM. This may lead to development of novel effective anti-GBM agents with significant clinical importance in GBM treatment due to DS have small molecular weight to pass the BBB and specific targeting GCSCs in GBM.

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Appendices

Appendix I

Peer-reviewed Publications

1. **Liu, P**., Kumar, I. S., Brown, S., Kannappan, V., Tawari, P. E., Tang, J. Z., Jiang, W., Armesilla, A. L., Darling, J. L. and Wang, W. (2013). "Disulfiram targets cancer stem-like cells and reverses resistance and cross-resistance in acquired paclitaxel-resistant triple-negative breast cancer cells." Br J Cancer 109(7): 1876-1885.

2. Yip, N. C.*, Fombon, I. S.*, **Liu, P**.*, Brown, S., Kannappan, V., Armesilla, A. L., Xu, B., Cassidy, J., Darling, J. L. and Wang, W. (2011). "Disulfiram modulated ROS-MAPK and NF-kappaB pathways and targeted breast cancer cells with cancer stem cell-like properties." Br J Cancer 104(10): 1564-1574. *Equal contribution

3. **Liu, P.**, Brown, S., Goktug, T., Channathodiyil, P., Kannappan, V., Hugnot, J. P., Guichet, P. O., Bian, X., Armesilla, A. L., Darling, J. L. and Wang, W. (2012). "Cytotoxic effect of disulfiram/copper on human glioblastoma cell lines and ALDH-positive cancer-stem-like cells." Br J Cancer 107(9): 1488-1497.

4. **Liu, P.**, Brown, S., Goktug, T., Channathodiyil, P., Kannappan, V., Hugnot, J. P., Guichet, P. O., Bian, X., Armesilla, A. L., Darling, J. L.

and Wang, W. (2013). "Reply: Cytotoxic effect of disulfiram/copper on human glioblastoma cell lines and ALDH-positive cancer-stem-like cells." Br J Cancer 108(19): 994.

5. **Liu, P.**, Wang, Z., Brown, S., Kannappan, V., Tawari, P.E., Jiang, W., Irache, J.M., Tang, J.Z., Armesilla, A.L., Darling, J.L., Tang, X., Wang, W. Liposome encapsulated Disulfiram inhibits NFκB pathway and targets breast cancer stem cells *in vitro* and *in vivo* Oncotarget 2014; Under review

Appendix II

Presentations

Oral presentations

Liu, P., Kumar, I.S., Brown, S., Kannappan, V., Tawari, P.E., Wang, Z., Tang, J.Z., Wang, W. Disulfiram targets cancer stem-like cells and reverses chemoresistance in an acquired paclitaxel resistant triple negative breast cancer cell line. UK PharmSci 2013, the Science of Medicines Conference, 2nd - 4th September 2013, Heriot-Watt University, Edinburgh UK.

Liu, P., Tang, J.Z., Wang, W. Repositioning of an old anti-alcoholism medicine into cancer therapeutics. UK PharmSci 2011, the Science of Medicines Conference, 31th August to 2nd September 2011, Nottingham, UK.

Poster presentations

Liu, P., Wang, W. AN ANTIALCOHOLISM DRUG BECOMES THE HOPE OF BRAIN CANCER PATIENTS has been selected to present in the House of Commons in Parliament on Monday 18 March 2013 for a poster scientific competition and exhibition.

Liu, P., Brown, S., Kannappan, V., Armesilla, A.L., Darling, J.L. and Wang, W. Disulfiram, an antialcoholism drug, targets breast cancer stem-

like cells. NCRI Cancer Conference, 4-7 November 2012, BT Convention Centre, Liverpool, UK.

Liu, P., Brown, S., Channathodiyil, P., Kannappan, V., Tang, J.Z., Armesilla, A.L., Darling, J.L. and Wang, W. Disulfiram, an antialcoholism drug, targets breast cancer and glioblastoma stem-like cells. EACR Annual Meeting, Barcelona 7th – 10th July 2012.

Liu, P., Brown, S., Armessilla, A.L., Darling, J.L., Cassidy, J. and Wang, W. Disulfiram – an old antialcoholism drug targeting breast cancer stem cells. NCRI Cancer Conference, 7-10 November 2010, Liverpool, UK.